

**ADVANCES IN THERMOCHEMOLYSIS:
TARGETED APPLICATIONS IN ANALYSIS OF
PLANT BIOMATERIALS**

FARZAD SHADKAMI

Advances in Thermochemolysis: Targeted Applications in Analysis of Plant Biomaterials

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Faculty of Science
Department of Chemistry
Memorial University of Newfoundland

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Abstract

Thermochemolysis (or thermally-assisted hydrolysis/methylation) is a unique *in situ* technique for analysis of a wide range of biomaterials, in their natural form, from plants material. It involves derivatization of analyte functional groups concurrent with mild thermal fragmentation of biomaterials and selective base-catalyzed cleavage of ester and ether bonds rather than the harsh thermal fragmentation and decomposition which is the case during the traditional pyrolysis. This thesis has targeted a number of special applications, all involving the chemical profiling of complex and different samples using trimethylsulfonium hydroxide (TMSH). Application of thermochemolysis-TMSH for the chemical profiling of needles of ozone fumigated pine trees showed that the concentrations of 3-hydroxybenzoic and 3,4-dihydroxybenzoic acids, useful as ozone damage markers, decreased with increased ozone dosage. It also revealed that the resin acids, anticopalic, 3-oxoanticopalic, 3 β -hydroxyanticopalic and 3,4-cycloanticopalic acid were prominent in the ozone-sensitive pine; however, only anticopalic acid was present in the ozone-tolerant clone. In another application, the chemistry of the antioxidant catechin was investigated under thermochemolysis conditions wherein both fully and partially methylated catechin in addition to a base-catalyzed epimerization product (i.e. methylated epicatechin) were identified. The polymeric structures of catechin-containing condensed tannins were also examined by thermochemolysis. A novel two-step methylation technique (TMS-diazomethane followed by thermochemolysis) was

developed which allowed for the observation, for the first time, of a dimeric catechin at MW = 540 amu.

A direct thermochemolysis solids injector device was constructed allowing the use of an injection port (instead of a pyrolyzer) for thermochemolysis-gas chromatography/mass spectrometry. The analytical usefulness of this thermochemolysis device was tested in direct comparison to a microfurnace pyrolyzer. It exhibited a similar chromatographic result for the thermochemolysis of standard catechin. Further, it was shown that this solids injector was suitable for real sample analysis containing a wide range of compounds including small phenolics, fatty acids, diterpene resin acids and flavonoids. In addition, chemical analysis of lignin macromolecules was investigated using the same direct thermochemolysis solids injector. This research work was the first to observe new dimeric thermochemolysis products (sylvatesmin and yangambin). These two lignin markers can be used to differentiate hardwood lignins from softwood lignins.

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List of Abbreviations

APPI-QqTOF-MS: atmospheric pressure photoionization quadrupole time-of-flight mass spectrometry

DCI: Direct Chemical Ionization

DP: degree of polymerization

DSI: direct sample introduction device

DTD: direct thermal desorption

Fully methylated catechin: Flavan, 3,3',4',5,7-pentamethoxy, trans

Fully methylated epicatechin: Flavan, 3,3',4',5,7-pentamethoxy, cis

GC: gas chromatography

HPLC: high-performance liquid chromatography

ID: internal diameter

MALDI: matrix assisted laser desorption ionization

MS: mass spectrometry

Partially methylated kaempferol: 3-Flavonol, 3',5,7-trimethoxy

Partially methylated catechin: 3-Flavanol, 3',4',5,7-tetramethoxy, trans

Partially methylated epicatechin: 3-Flavanol, 3',4',5,7-tetramethoxy, cis

Partially methylated epigallocatechin: 3-Flavanol, 3',4',5,5',7-pentamethoxy, cis

Partially methylated gallocatechin: 3-Flavanol, 3',4',5,5',7-pentamethoxy, trans

Partially methylated quercetin: 3-Flavonol, 3',4',5,7-tetramethoxy

PC: Procyanidin

PD: prodelphinidins

Py: pyrolysis

Py-GC-MS: pyrolysis gas chromatography mass spectrometry

RT: retention time

SPME: solid phase microextraction

TBAH: tetrabutylammonium hydroxide

TEAAc: tetraethylammonium acetate

THM: thermally assisted hydrolysis and methylation

TMAAc: tetramethylammonium acetate

TMAH: tetramethylammonium hydroxide

TMPAH: trimethylphenylamine hydroxide

TMS-diazomethane: trimethylsilyl diazomethane

TMSH: trimethylsulfonium hydroxide

WSCP: whole sample chemical profiling

Chapter 1: Introduction and Overview

1.1 Analytical Pyrolysis

1.1.1 General

Analytical pyrolysis refers to thermal decomposition or transformation of compounds in the absence of oxygen at temperatures from 450°C to 700°C. Analytical pyrolysis is applicable to organic compounds which are not volatile or cannot be brought into solution; therefore, they are not suitable for direct analysis by common analytical techniques such as gas or liquid chromatography. Compounds such as polymers are candidates for analytical pyrolysis wherein thermal energy is applied to cleave the polymeric bonds resulting in degradation products that are volatile enough for gas chromatography analysis. Analytical pyrolysis is different from applied pyrolysis. Applied pyrolysis is concerned with the production of chemicals, for example, the industrial production of carbon from the pyrolysis of coconut shells.

Thermochemolysis is complementary to analytical pyrolysis wherein heat primarily is used to drive a reaction between a methylating reagent and the analyte's acidic functional groups in order to decrease the polarity of the analyte. At the same time, heat is used to hydrolyze ester or ether bonds and to a lesser extent induce thermal fragmentation of the analyte.

Pyrolysis/thermochemolysis is mainly performed *in situ* using pyrolysis gas chromatography mass spectrometry (Py-GC-MS). It can also be done offline [1] but the procedure involves more sample handling and products are exposed to air and moisture. Pyrolysis/thermochemolysis is normally performed by rapid heating (flash pyrolysis) or by temperature-programmed heating.

1.1.2 Py-GC-MS

Py-GC-MS is a technique used for the decomposition/transformation, separation, detection and identification of mixtures of compounds. Pyrolysis is used to thermally decompose or fragment individual compounds or polymers into volatile fragments/compounds. Pyrolysis products (pyrolysates) are transferred to the injection port of a gas chromatograph via a pyrolysis transfer line. A gas chromatograph is used for separation of the pyrolysate, and a mass spectrometer is used for the detection and identification of product compounds. Analytes are separated based on their different affinities for the stationary and mobile phase in a chromatographic column. Based on the boiling points of compounds, the partition is affected by the column temperature. In GC, the mobile phase is an inert gas (e.g. helium) while the stationary phase is a silica-bonded high molecular weight polymer deposited on walls of a capillary column. In GC, each compound is characterized by its specific retention time. Retention time is a variable for qualitative analysis. This variable is measured relative to the elution time of an unretained solute. The data related to the retention time is recorded in a graph called

chromatogram. Since many compounds may possess the same retention time the use of another detection system is necessary. After compounds are separated in a GC, they are eluted from the column and passed into a mass spectrometer where the analytes are discriminated based on their unique electron impact mass spectra or mass to charge ratio (m/z) of the ions they produce.

A Py-GC-MS is composed of a pyrolyzer, a pyrolyzer transfer line, an injection port, a column, GC-MS interface and a mass spectrometer which is made up of an ionization source, a mass analyzer and an ion detector as seen in Fig. 1.1 [2-4]. The pyrolyzers and the pyrolyzer transfer line are critical parts of a Py-GC-MS as noted in Section 1.1.2.2.

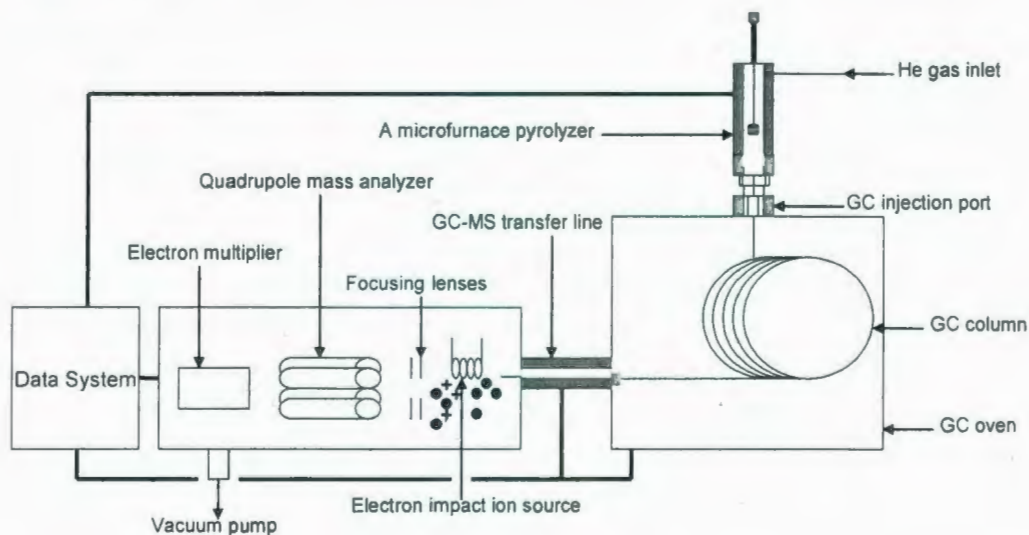


Fig. 1.1 Schematic set-up of a Py-GC-MS

1.1.2.1 Pyrolyzers

Pyrolyzers can be categorized into three types, namely, microfurnace pyrolyzers, Curie-point pyrolyzers and filament pyrolyzers. In a microfurnace pyrolyzer, the sample is dropped into the small hot quartz furnace by using a sample holder (see Fig. 1.3). The sample can be exposed to a pyrolysis temperature for a desired time. A carrier gas is used to sweep the pyrolysis products onto a chromatographic column for analysis. Unlike the other two pyrolyzers, the sample is not exposed to high temperatures before the pyrolysis stage since the sample is held above the furnace at ambient temperature before the sample drop (Fig. 1.3). In a Curie-point pyrolyzer the sample is placed onto a ferromagnetic wire and the wire is inserted into the pyrolyzer. The wire is heated rapidly using a high frequency induction coil until the Curie-point temperature of the metal is reached. The choice of pyrolyzer temperature is limited and depends on the Curie-points of the available metals. In filament pyrolyzers, quartz tubes are used to hold the sample before being inserted into a coil filament (see Fig. 1.2). The filament is heated by a rapid heating current to the filament [5].

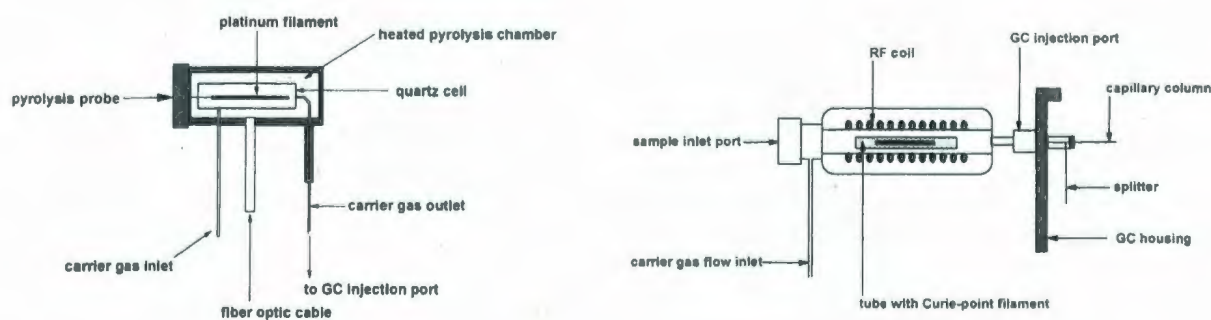


Fig. 1.2 Schematic of a filament pyrolyzer (left) and a Curie-point pyrolyzer (right)
(from [5])

Off-line pyrolysis can also be employed for producing pyrolysates as has been demonstrated for plant biomaterials [1-2]. A small amount of the sample is placed on a small plug of quartz wool in a quartz tube. The quartz tube was positioned inside the coil of pyroprobe which is then heated in an inert nitrogen atmosphere. The pyrolysis products are swept into and are condensed on a quartz tube that is cooled with liquid nitrogen. After pyrolysis, the products are rinsed with a suitable solvent and analyzed. Off-line pyrolytic analysis is suitable in situations where the GC injector can not be "tied-down" to a pyrolyzer interface, or, analysis of products is carried out by other techniques, e.g. high resolution MS.

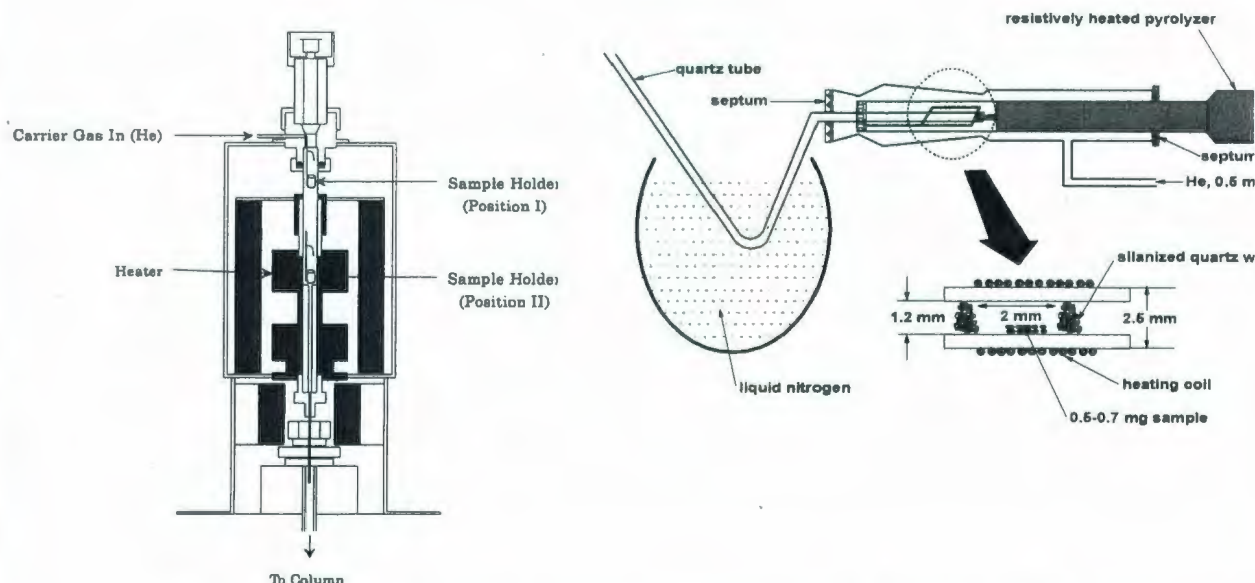


Fig. 1.3 Schematic of a modified microfurnace pyrolyzer (left) and an off-line pyrolyzer system (right) (from [5])

1.1.2.2 Pyrolyzer Transfer Line

A pyrolyzer transfer line plays a critical role in pyrolysis. It has been shown that there is a discrimination of high boiling point or high molecular weight products in transfer from a pyrolyzer to a GC injection port [6]. One of the techniques used to reduce this discrimination is the use of an in-column pyrolysis device. In this approach, one end of the tubing is connected to a GC column and the other connected to GC injection port. Heat is produced by passing an electric current pulse through the stainless steel tubing. Because the sample holder is located inside the GC oven unwanted condensation of products is minimized. It was shown that high molecular weight fatty acid methyl esters produced by thermochemolysis in the presence of TMAH were discriminated when a conventional pyrolysis unit was employed [7-8].

The temperature control of the pyrolysis transfer line, GC injection port and the GC-MS interface are important in the rapid transfer of fragments, especially large fragments to the mass analyzer and the detector.

1.1.3 Biomaterials by Py-GC-MS

Py-GC-MS has been previously applied to the analysis of a wide range of biomaterials including resin acids [9], fatty acids [10], flavonoids [11], and lignins [12].

In pyrolysis, resin acids are degraded into smaller and more volatile neutral fragments. Pyrolysis of abietic acid, dehydroabietic acid and methyl dehydroabietate at 800 °C yielded toluene, styrene, indene, naphthalene, 2-methylnaphthalene, 2-vinylnaphthalene and phenanthrene [9]. In another study, when pyrolysis was performed at 600-800 °C manila copal, a protective resin acid-containing layer for painting, showed the presence of methyl-isopropylnaphthalene, a known thermal degradation product of abietic acid. Other resins, also used as a protective layer for painting, showed distinctive pyrolysis products. Colophony showed the presence of a terpenic ketone and also the presence of a manila copal pyrolysis product (i.e. thermal degradation products of manila copal). The pyrolysis products of Venice Turpentine were identified and shown to be the same as the abietic acid pyrolysis products (m/z 184 and 169). Elemi was identified by the presence of elemicine or 3,4,5-trimethoxyallylbenzene, and Dammer identified by the presence of two isomers of sesquiterpene ($m/z = 202$) [13]. In another study, abietic acid's degradation products and those of its isomers were obtained by pyrolysis at 610°C of Colophony [14]. It was suggested that abietic acid isomers are interconvertible through isomerization of the double bond under pyrolysis conditions. Natural resins applied in painting were also studied by pyrolysis-silylation [15]. In this technique, hexamethyldisilazane was used to derivatize the resin acids prior to pyrolysis. Several trimethylsilyl esters of resin acids were reported in the pyrolysates.

A number of studies have been conducted on the pyrolysis of fatty acids [10,16-17]. In one study, the carboxyl groups of fatty acids were converted to the corresponding sodium salts to prevent evaporation of the acids under pyrolysis conditions [10]. The pyrolysis of the sodium salt of hexadecanoic acid yielded homologous series of *n*-alk-1-enes and *n*-alkanes up to C₁₅. The initiation step in the thermal dissociation of the model compounds was proposed to be C-C bond cleavage because of the lower bond dissociation energy of C-C bonds (334 KJ/mol) relative to the dissociation energies of C-H bonds (420 KJ/mol) and C-O bonds (340 KJ/mol). Pyrolysis was also applied to the analysis of lipids [17] wherein hexamethyldisilazane (HMDS) was used as a derivatization reagent prior to pyrolysis. This study was able to discriminate between egg tempera and siccative oils in paint layers.

Py-GC-MS has been also applied in analysis of flavanoids [11, 18]. In the pyrolysis of catechin at 600 °C, catechol was found as the major fragment [11], while pyrogallol was the main fragment of galocatechin [18].

Polymeric structures of lignins have been extensively investigated by Py-GC-MS [12, 19-22]. Alder [12] suggested three monomeric components of a highly cross-linked lignin structure including *p*-coumaryl alcohol (or *p*-hydroxy-cinnamyl alcohol), coniferyl alcohol (or guaiacyl alcohol), and sinapyl alcohol (or syringyl alcohol) and their aldehyde derivatives. It was found that these structures vary between tree species, for example,

hardwood, softwood and grass lignins contain different lignin components. Softwood lignins contain coniferyl derivatives, hardwood lignins contain coniferyl and sinapyl derivatives, and grass lignins contain *p*-vinylphenol [19].

In a recent study [21], the pyrolysis behavior of milled wood lignin isolated from Japanese cedar wood was compared with that of various guaiacyl-types of lignin model dimers. This study was special in that it was performed by off-line pyrolysis at 400 °C followed by HPLC analysis. The major pyrolytic pathways for both dimer and milled wood lignin involved the cleavage of C_β-O with the formation of cinnamyl alcohol-type structure (β-ether linkage) and C_γ-elimination with the formation of a stilbene-type structure (β-aryl linkage). In another study, Py-GC-MS of lignins revealed the presence of a unique 5-hydroxyguaiacyl unit by the following products; 5-vinyl-3-methoxycatechol, 3-methoxycatechol, and 5-propenyl-3-methoxycatechol; however, the concentrations of these compounds were lower than the guaiacyl and syringyl-related products [21]. There are other publications which suggest different original substructures for lignin based on their pyrolytic products. Kuroda [22] proposed a β-5 substructure of a 2-arylcoumaran lignin model compound. One disadvantage of conventional direct pyrolysis is that the pyrolysis products were not derivatized; consequently, the fragments containing polar functional groups exhibited poor chromatographic behavior. Sonoda et al. [23] improved this by acetylation of functional groups in the lignin prior to pyrolysis.

One advantage of this technique was the prevention of secondary formation of cinnamaldehyde from the corresponding alcohol formed during pyrolysis.

1.2 Thermochemolysis

1.2.1 General

The origin of thermochemolysis goes back to 1967 when it was used for analysis of fatty acids [24-25]. Thermochemolysis is complementary to conventional direct pyrolysis and can provide more definitive structural information of the original composition of the sample. In thermochemolysis, heat is primarily used to drive the reaction between the methylating reagent and the acidic functional groups of the analytes resulting in the formation of the methyl esters of the carboxylic acids and the methyl ethers of the alcohols. At the same time, heat assists in base-catalyzed cleavage of ester and ether bonds and to a lesser extent thermal fragmentation. Since a wide range of biomaterials contain carboxylic acid and phenolic functional groups, thermochemolysis promises a bright future for routine analysis of biomaterials. Thermochemolysis procedures show a series of advantages including smaller sample size, minimum sample treatment and handling, and faster analysis time. Thermochemolysis has been called by number of scientific names including thermally assisted hydrolysis/methylation (THM), reactive pyrolysis, pyrolysis-methylation, and simultaneous pyrolysis and methylation (SPM). One of the major advantages of thermochemolysis over conventional direct

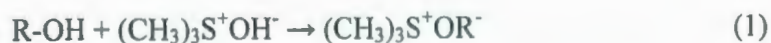
pyrolysis is that it overcomes problems due to the formation of polar products in direct pyrolysis. Polar compounds are susceptible to adsorption onto wall surfaces in the pyrolyzer, liners in the injection port, and the chromatographic column which results in contamination, peak tailing and poor reproducibility.

A number of reagents exist for thermochemolysis including tetramethylammonium hydroxide (TMAH), trimethylsulfonium hydroxide (TMSH), tetrabutylammonium hydroxide (TBAH) [26] and tetramethylammonium acetate (TMAAc) [27]. The most common reagent is TMAH which is strongly basic and has good methylating ability; however, it carries some disadvantages. TMAH can result in the production of multiple peaks due to base-catalyzed isomerization of base-labile compounds, e.g. polyunsaturated fatty acids. Thermochemolysis in the presence of TMAH does not distinguish fatty acids from fatty-acid esters (i.e. triacylglycerols) while it has been shown that TMAAc, because of its lower alkalinity, is able to methylate fatty acids, but is not able to break the fatty acid ester bonds and methylate them [28]. Thermochemolysis in the presence of TBAH is able to discriminate between experimentally added butyl groups and originally existing methyl groups while methylating agents such as TMAH and TMSH do not possess this advantage.

1.2.2 Thermochemolysis-TMSH

Like TMAH (tetramethylammonium hydroxide), TMSH (trimethylsulfonium hydroxide) is an organic base and is routinely synthesized from trimethylsulfonium iodide [29]. It has had limited usage in thermochemolysis only because TMAH was a popular reagent and until now was easier to obtain from supply companies. TMSH has been used as an alternative methylating reagent to TMAH in a number of recent studies for analysis of lipids [30-39] and phenols and acidic herbicides [40]. Because it is sensitive to moisture and carbon dioxide, one needs to purchase it as a methanolic solution (0.25 M) sealed in a septa dispensing bottle. This is a disadvantage for sample handling. Also, samples for analysis with TMSH need to be dry. Unlike TMSH, both aqueous and methanolic solutions and the solid form of TMAH are commercially available.

The suggested mechanism for the reaction of phenolics (or carboxylic acids) with TMSH is as follows:



In cases where ester or ether bonds are present, the analyte first undergoes hydrolysis with the subsequent formation of a salt. In contrast to TMAH [41], this

reagent has been shown to be suitable for the analysis of alkaline- or heat-sensitive samples because the methylation step (2) requires lower thermochemolysis temperatures. This decreases the likelihood of base isomerization of polyunsaturated fatty acids [30]. One of the objectives of this thesis is to explore the applicability of TMSH under different reaction conditions for the analysis of a wide range of compounds many of which have not been investigated by other researchers.

1.2.3 Thermochemolysis Devices

All three types of pyrolyzers (described in Section 1.1.2.1) have been used for thermochemolysis. Some examples are as follows: Nierop et al. [42] used a Curie-point pyrolyzer for thermochemolysis of condensed tannins and Garnier et al. [43] used a filament pyrolyzer for analysis of polyphenols from modern and archaeological vine materials. Also, Estevez and Helleur [30] used a microfurnace pyrolyzer for thermochemolysis of marine lipids. All types of pyrolyzers are suitable for thermochemolysis; however, the solvent and excess methylating reagent need to be purged in advance to prevent them from entering the analytical GC column.

Although there are many workers using all three types of pyrolyzers, the limitations of each pyrolyzer cannot be neglected. The limitations of all pyrolyzers, which are external devices to the GC, are related to the discrimination of high boiling point or high molecular weight products in the transfer from the pyrolyzer to the GC

injection port [6]. As well, the Curie-point and filament pyrolyzers have the additional disadvantage of subjecting the sample/TMAH to premature heating before thermochemolysis. This does not occur when using the microfurnace as previously explained.

One solution to this problem is the use of a GC injection port since some thermochemolysis-TMSH approaches use 300°C-400°C temperatures, which can be accommodated by the GC injection port. There have been commercial solids injector devices used to introduce samples into the GC injection port and have the potential for usage in thermochemolysis. These devices include a solid injector, Keele [44], a solid phase microextraction (SPME) fiber [45], a direct sample introduction device (DSI) [46], a syringless injector [47], and a solids injector commercially available from SGE, Australia. The commercially available solids injector is a syringe-like device, consisting of a metallic rod sample holder inside a syringe needle. The disadvantage of this device is severe blockage after just a few uses. In the Keele injector, a sample is sealed in a glass ampule and released by breaking the ampule in the GC injection port. The DSI device requires a modified GC injector wherein sample is introduced via a sample holder. In a syringless injector, a glass vial moves vertically into and out of the injection port by controlling the carrier gas flows. The Keele injector is labor intensive, and DSI and syringless injector require a major modification of the GC injector port or purchase of a

modified injection port. SPME was used for thermochemolysis, but most likely the SPME fiber will be damaged after a few usages in alkaline conditions.

One of the objectives of this thesis is to construct a simple but practically useful solids injector for thermochemolysis of natural compounds.

1.3 Thermochemolysis of Biomaterials in Plants

The major theme of this thesis is to develop rapid thermochemolytic approaches to characterize plant materials and their extracts using selected methylating reagents. The following is an extensive literature review of past studies undertaken on resin acids/terpenoids, fatty acids, condensed tannins and lignins.

1.3.1 Resin Acids

Resin acids belong to a class of compounds commonly found in trees and shrubs called terpenoids. They act as protectants and wood preservatives which are located near resin ducts in trees from temperate coniferous forests. Terpenoids are multiples of the isoprene unit (a branched 5-carbon unit) and are classified into monoterpenoids (C_{10}), sesquiterpenoids (C_{15}), diterpenoids (C_{20}) and triterpenoids (C_{30}). Resin acids usually refer to tricyclic or bicyclic diterpenoids (C_{20}) that are natural products with a carboxylic acid functional group on the parent hydrocarbon derived from isoprene units. The labdane type of resin acids, bicyclic diterpenoids, are secondary metabolites, which have been reported to have a broad range of biological activities [48-49]. The common

tricyclic resin acids are abietic (2), neoabietic (4), palustric (1) and pimaric acids (5), and the common bicyclic resin acid is anticopalic acid (6). These and a few less common structures are given below.

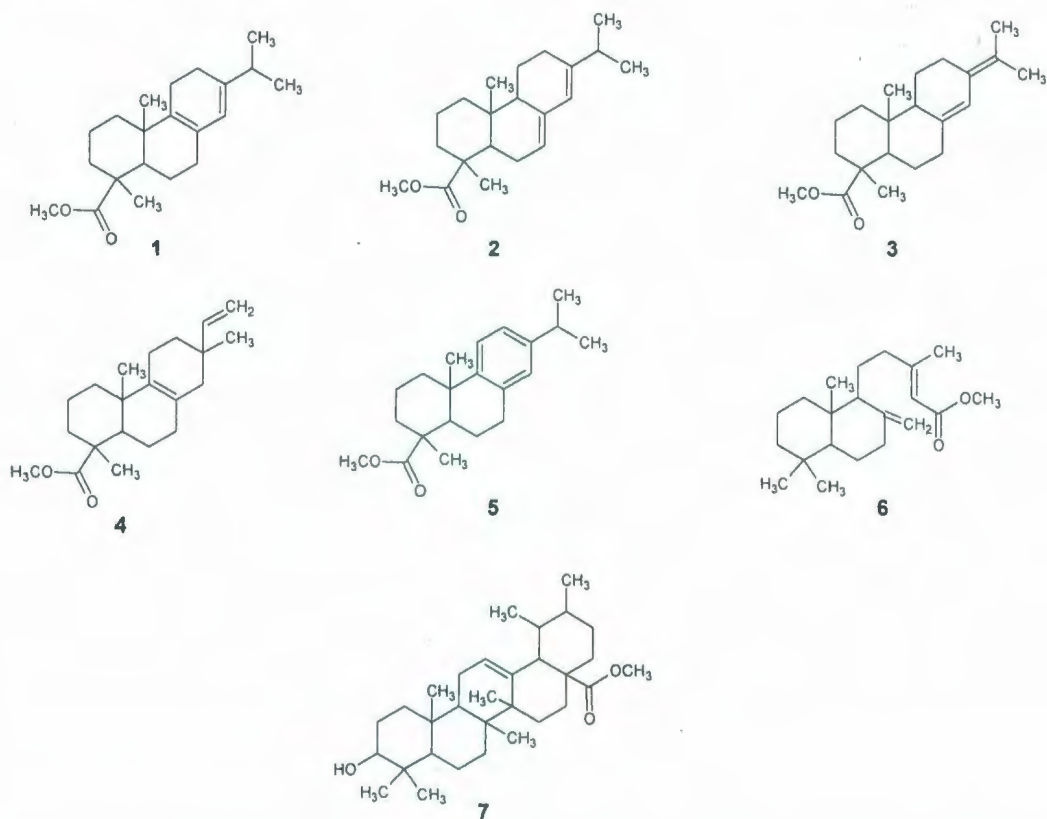


Fig. 1.4 Chemical structures of resin acid methyl esters of (1) palustric acid (2) abietic acid (3) neoabietic acid (4) pimaric acid (5) dehydroabietic acid (6) anticopalic acid (7) ursonic acid.

Different techniques have been used for analysis of resin acids including Fourier transform infrared (FT-IR) [50], NMR [51], high-performance liquid chromatography (HPLC) [52], and GC-MS [53].

The separation and identification of resin acids were thoroughly studied by Zinkel et al. [53-56]. Resin acids of needle and cortex tissues from *Pinus strobus*, *P. nigra* and *P. sylvestris* were isolated, identified, and quantitatively determined by gas chromatography [56]. Needles were extracted with diethylether and resin acids were separated and methylated with diazomethane. The most common tricyclic diterpenoid resin acids found were abietic, neoabietic, palustric and dehydroabietic. The most common bicyclic resin acid was anticopalic acid; however, a large amount of 3-ketoanticopalic acid was also found. The resin acids of cortex, in contrast to needles, consist of primarily tricyclic abietane and pimarane types. Zinkel et al. [51] reported the presence of 3-oxoanticopalic acid, 3 β -acetoxoanticopalic, 3 β -hydroxyanticopalic acid and the 8 α -hydroxy derivative of anticopalic acid in the needles of *Pinus strobus* and the structures were determined by NMR. Some resin acids exhibited relatively unstable behavior under heating and light conditions. For example, abietane structures are known to isomerise easily to form abietic acid. Abietic acid has a tendency to oxidize in air to the aromatic dehydroabietic acid.

Thermochemolysis has been applied for the analysis of the resin acids either present in the plant tissue or contained in art and paintings which used resins as a base [57-58]. It was found in many cases that thermochemolysis provides a useful resin acid profile using TMAH [57-59]. Resin acid methyl esters were easily produced *in situ* and thermochemolysis did not result in thermal degradation or isomerization of products. The

exception is for resin acids with hydroxyl and/or carbonyl functionality. Resin acids with hydroxyl and/or carbonyl functionality undergo side reactions with TMAH, which result in the formation of unusual nitrogen-containing products [58].

Thermochemolysis was also applied to varnish samples from art works [57]. Two oxidized derivatives of methyl dehydroabietic acid, namely 7-oxo-dehydroabietic acid methyl ester and 7-oxo-15-hydroxy-dehydroabietic acid methyl ester were identified by thermochemolysis in the presence of TMAH at 600 °C. In another study [59] free labdane type diterpenoids and a cross-linked fraction of polycommenic acid were observed in two natural resins of copals and sandarac by thermochemolysis-TMAH at 600 °C. The limitation of this work was the secondary degradation reactions of diterpenoid resin acids which complicated the interpretation of mass spectra. Triterpenoid resins including ursolic acid and methyl ursonate were also studied by thermochemolysis in the presence of TMAH [60]. The paper was impressive as a result of the large number of MS fragmentation pattern interpretations reported and the number of mass spectra of triterpenoid resin derivatives given. The thermochemolysis technique was also applied in characterization of aged triterpenoid varnishes [60].

Diterpenoid resins from larch and pine trees and the varnish of a 200-year-old paint sample were analyzed by thermochemolysis-GC-MS in the presence of TMAH [61]. The main resin acids were found as highly oxidized abietic acids including 15-

hydroxy-7-oxodihydroabietic acid. It was proposed that palustric acid, abietic acid and neoabietic acid are interconvertable, and dehydroabietic acid is the side-product of abietic acid under thermochemolysis conditions. It was shown that six abietic acid derivatives resulted from thermochemolysis of dehydroabietic acid.

In another study, resin acids in violin varnishes including colophony, sandarac, and manila copal were investigated by thermochemolysis-GC-MS [62]. In colophony varnish, methyl esters of pimaric, isopimaric, palustric, dehydroabietic, abietic, neoabietic, and 7-oxo-dehydroabietic acids were found. It was suggested that pimaric resin acids are relatively stable to isomerization/oxidation reactions under TMAH conditions. The presence of 7-oxodehydroabietic acid indicates that the colophony resin was aged since this marker is an oxidation product of abietic acid. Elemi resin was characterized by identifying the triterpenoid compounds β -amyrin and α -amyrin as well as some sesquiterpenic compounds. Moreover, a real sample of Marchi varnish was characterized by the presence of chemical markers of colophony including methyl esters of pimaric, dehydroabietic, 7-methoxytetrahydroabietic and 7-oxodehydroabietic acids, which indicated that the natural resin used in the formulation of this varnish was colophony.

1.3.2 Fatty Acids

Fatty acids are an important component of lipids (fat-soluble components of living cells) in plants, animals, and microorganisms. They are carboxylic acids (saturated or unsaturated) with an unbranched aliphatic chain and an even number of carbon atoms if they are from plant sources. High-performance liquid chromatography (HPLC) has been commonly used for analysis of fatty acids. The most common sample preparation is solvent extraction with non-polar solvents such as hexane, chloroform and diethyl ether. For fatty acid analysis, the LC stationary phase is usually a reverse-phase system consisting of alkyl chains of various lengths bonded onto a silica base. The mobile phases used are typically water mixed with a less polar solvent such as acetonitrile or methanol. Mass spectrometry coupled with HPLC is the most recent technique for their detection [63]. More complex fatty acids structures can be investigated by tandem mass spectrometry (MS) wherein a precursor ion selected by the first MS, is subjected to fragmentation by collision with inert gas molecules. As a result of collision-induced dissociation, product ions are formed from which the structure of the precursor ion can be elucidated using the second MS. In tandem mass spectrometry, the ionization and fragmentation properties of fatty acids are improved by derivatization to aminoethyltriphenylphosphonium derivatives [64].

Gas chromatography (GC) has been the most widely used technique for the analysis of fatty acids [65]. Fatty acids are usually converted to their more volatile derivatives such as methyl esters and trimethylsilyl (TMS) derivatives.

Thermochemolysis has been successfully used for profile analysis of fatty acids and fatty acid-containing lipids [66-71]. When fatty acids are bound as in triacylglycerides (via ester bonds), they can be converted to their free methyl esters as a result of saponification followed by methylation. Different reagents were compared for thermochemolysis of fatty acids and triacylglycerides, such as TMAH, TMAAc, TMSH and trimethylphenylamine hydroxide (TMPAH). One study showed that TMPAH provided the highest yields of fatty acid methyl esters [66]. Cappitelli et al. [67] investigated the effect of temperature on reproducibility of the results of fatty acid methyl ester formation. They described the use of thermochemolysis-GC-MS to measure differences in the fatty acid composition of dried linseed, linseed, poppy, and walnut oil. It was shown that a pyrolysis temperature of 610°C had a higher degree of reproducibility when using palmitate/stearate ratios as a standard.

Thermochemolysis-GC-MS in the presence of TMAH was applied to the analysis of biological materials collected from the atmosphere [68]. Eukaryotic organisms could be differentiated from prokaryotic microorganisms collected by their polyunsaturated fatty acid content. The most characteristic fatty acid methyl ester biomarker was C18:3

present in eukaryotic cells. Whole cell samples were also differentiated from proteins based on the lack of fatty acid methyl ester biomarkers in the protein material. In a similar study, thermochemolysis-GC-MS was applied to the analysis of whole-cell bacterial samples whereby 15 bacteria were differentiated [69]. Hardell and Nilvebrant [70] discriminated between free and esterified fatty acids using two different reagents, TMAAc and TMAH. TMAAc was chosen as a selective reagent for the methylation of free fatty acids, while TMAH was found to be efficient in methylating all fatty acids present, both as free acids and in different esters.

Thermochemolysis-TMAH was shown to bring about isomerization and/or degradation of polyunsaturated fatty acids such as linoleic (C18:2) and linolenic acid (C18:3) residues [41]. On the other hand, trimethylsulfonium hydroxide (TMSH) was shown to be a more suitable reagent for analysis of complex and less stable marine lipids since it does not cause extensive isomerization of polyunsaturated fatty acids [31]. In a marine chemistry study of free fatty acids, phospholipids, and triacylglycerides, these individual classes were successfully separated on a thin layer TLC rod. The developed rod containing these lipid bands (with TMSH) was successfully profiled for fatty acid content using a vertical furnace pyrolyzer coupled to a GC-MS [30]. It was further discovered that the use of TMSH along with silica surface minimized degradation of lipids.

Finally, a new methylating reagent has recently been proposed. Dimethyl carbonate in the presence of a catalyst has been used as thermochemolysis reagent for pyrolysis transmethylation of triacylglycerides at 700°C [71]. In this study, Ti-doped MCM-41 mesoporous materials, titanium silicate and TiO₂ nanopowders were compared as catalysts with dimethyl carbonate. It was found that Ti-MCM-41 was most effective in producing fatty acid methyl esters by Py-GC-MS, but the conditions caused double bond isomerisation of linoleic acid when compared to TiO₂ nanopowders. The main advantage of dimethyl carbonate is its non-toxic and non-corrosive properties.

1.3.3 Catechins and Condensed Tannins

Condensed tannins are distributed all over the plant kingdom. They are commonly found in both gymnosperms as well as angiosperms. After cell breakdown and death, tannins are active in metabolic effects. They may play a role in inhibiting microbial activity, thus resulting in the natural durability of the wood. The traditional usage of tannins by human is in the tanning process for making leather, which is related to protein-binding property of tannins. The monomeric units of the condensed tannins are called "catechins" which include catechin, epicatechin, epigallocatechin and gallocatechin, all belonging to a family known as flavan-3-ols (or 3-flavanols). These catechins are also the main monomeric units present in condensed tannins. Condensed tannins can vary in the number of hydroxyl groups in the B-ring. Procyanidin (PC)

condensed tannins contain two hydroxyl groups (e.g. catechin (1) and epicatechin (2) monomers), while prodelphinidin (PD) condensed tannin contain three hydroxyl groups (e.g. epigallocatechin (3) and gallocatechin monomers) in their B-ring (see Fig. 1.5).

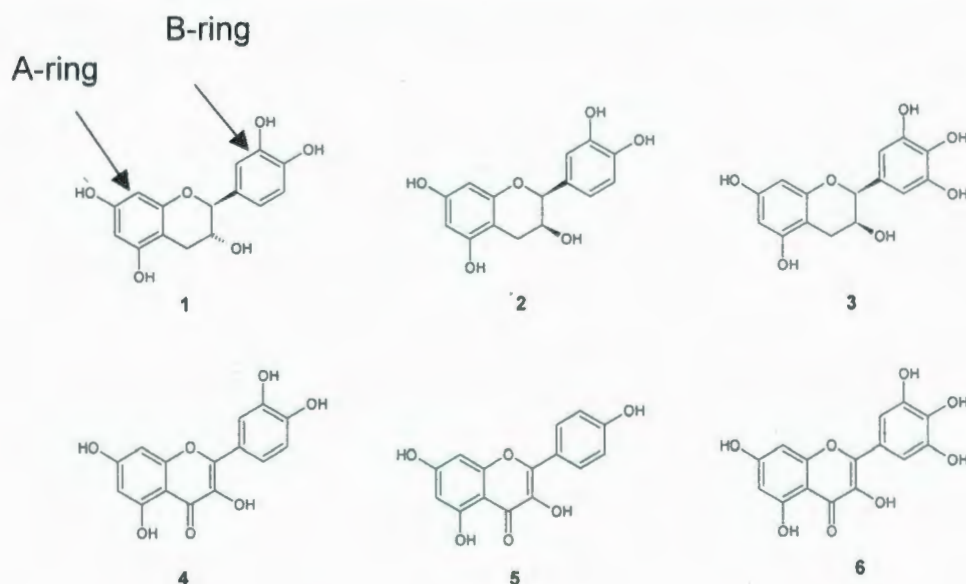


Fig. 1.5 Chemical structures of common flavanoids (1) catechin (2) epicatechin (3) epigallocatechin (4) quercetin (5) myricetin (6) kaempferol.

Since thermochemolysis occurs under basic conditions an understanding of the behavior of analytes under these conditions is necessary. The behavior of catechin, epicatechin and procyanidin B₃ (catechin-(4 α -8)-catechin) were studied under basic conditions [72-74]. Catechinic acid and a unique catechinic acid stereoisomer resulted from the base-catalyzed reaction of catechin and epicatechin [72-73]. It was shown that

both rearrangement and dimerization reactions followed a similar mechanism in the base-catalyzed reaction of both monomers. Procyanidin B₃ at pH 12 and 40°C gave a double-linked catechinic acid-catechin dimer, catechinic acid and a catechinic acid stereoisomer. All the products are believed to be produced through the formation of a quinone methide intermediate. In base-catalyzed reactions of procyanidin, the terminal unit retained its chemical structure [74].

One of the common techniques in characterization of condensed tannins is the acid-catalyzed cleavage between flavan-3-ol units in the presence of phloroglucinol or benzyl mercaptan [75]. The thiolysis study showed that condensed tannins from *Lotus corniculatus* and *L. pedunculatus* are composed of procyanidin and prodelphinidin units with *L. pedunculatus* having a higher proportion of prodelphinidin units than *Lotus corniculatus* condensed tannins. Sun et al. [76] reported tandem mass spectrometry of B-type procyanidins in wine. Procyanidin B₂ and B-type dehydrodiccatechins (dimeric flavan-3-ols) gave different fragmentation in tandem mass spectrometry because of the presence of different C-C interflavan linkage. Condensed tannins and catechin were also investigated using pyrolysis GC-MS. Catechol was the main pyrolysis fragment for both catechin and condensed tannins [11]. Zeeb et al. [77] developed a liquid chromatography atmospheric pressure chemical ionization (LC/APCI-MS) method for separation of twelve catechins including catechin, epicatechin, gallocatechin, epigallocatechin, and their gallate derivatives in green and black tea infusions. In this work, it was observed

that a fragment ion at m/z 139 provided a unique fingerprint for catechin content, which was produced by retro Diels-Alder reaction.

In situ thermochemolysis of condensed tannins, catechin, epicatechin and epigallocatechin has been investigated [78-79, 42-43]. All above studies reported A-ring thermochemolysis products such as 1,3,5-trimethoxybenzene (7) and 2,4,6-trimethoxytoluene (3). However, only Nierop et al. [42] and Galletti and Bocchnini [78] found additionally the pyrolysate 2-ethyl-1,3,5-trimethoxybenzene (4) as an A-ring THM product (see Fig. 1.6). These A-ring markers cannot discriminate procyanidin from prodelphinidin because procyanidin and prodelphinidin have the same A-ring structure. Nierop et al. [42] and Galletti and Bocchnini [78] found a small amount of 1,2-dimethoxybenzene (5) as the main B-ring product of catechin/epicatechin whereas Garnier et al. [43] reported a significant amount of 3,4-dimethoxybenzoic acid methyl ester (2) and 3,4-dimethoxystyrene as B-ring products of catechin and epicatechin. Nierop et al. [42] reported 1,2,3,5-tetramethoxybenzene as a prodelphinidin marker and 1,2,4-trimethoxybenzene (6) as a procyanidin marker. As well, Garnier et al. [43] and Nierop et al. [42] reported 1,2,3-trimethoxybenzene (1), 3,4,5-trimethoxytoluene (8) and 3,4,5-trimethoxybenzaldehyde as epigallocatechin markers.

In a study by Nierop et al. [42], statistical analyses of the thermochemolysis products coupled to confirmational NMR data of tannins was used to investigate the

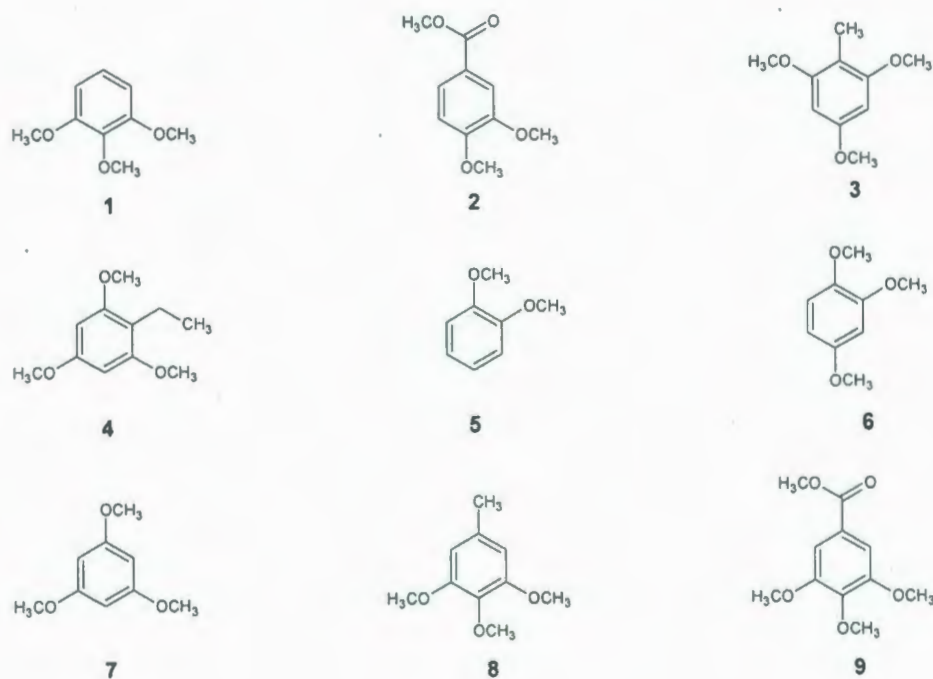


Fig. 1.6 Flavan-3-ol thermochemolysis products (1) 1,2,3 trimethoxybenzene (2) 3,4-dimethoxybenzoic acid methyl ester (3) 2,4,6-trimethoxytoluene (4) 2-ethyl-1,3,5-trimethoxybenzene (5) 1,2-dimethoxybenzene (6) 1,2,4-trimethoxybenzene (7) 1,3,5-trimethoxybenzene (8) 3,4,5-trimethoxytoluene (9) 3,4,5-trimethoxybenzoic acid methyl ester. From [42] and [43]

correlations between the B-ring structural units (i.e. procyanidin vs prodelphinidin) and the di-, tri- and tetra-methoxybenzene fragmented products. The correlation was performed based on the assumption that all 1,2-dimethoxybenzenes were assigned as procyanidin B-ring products, and all 1,2,3-trimethoxybenzenes were assigned as prodelphinidin B-ring products. An example of this analysis include 1,2,4-trimethoxybenzene which was classified as a procyanidin B-ring product and 1,2,3,4-

tetramethoxybenzene which was assigned as a prodelphinidin B-ring product. Furthermore, based on the correlation analysis, they proposed two mechanisms for the formation of B-ring thermochemolysis products that explain the cleavage of C1'-C2 and C2-C3 linkages. Real sample applications of this study were demonstrated in condensed tannin extracts of tree species and shrub.

1.3.4 Lignins

Lignin is the most abundant phenolic compound in nature and it is the main constituent of cell walls in plants after cellulose and one of the most difficult biopolymers to analyze. Structural information on lignin is fragmentary due to its insolubility and the heterogeneity of its subunit structures and linkages. The main monomeric components of the polymeric structure of lignin were proposed as phenylpropenyl units (C₆-C₃) [80-81]. This includes *p*-coumaryl alcohol (**1**) (or *p*-hydroxy-cinnamyl alcohol), coniferyl alcohol (**2**) (or guaiacyl alcohol) and sinapyl alcohol (**3**) (or syringyl alcohol) and their aldehyde derivatives. These structures are shown in Figure 1.7.

Lignins and lignin model compounds have been studied by different techniques including NMR [82], matrix assisted laser desorption ionization (MALDI) [81], atmospheric pressure photoionization quadrupole time-of-flight mass spectrometry (APPI-QqTOF-MS) [83] and polarography [84].

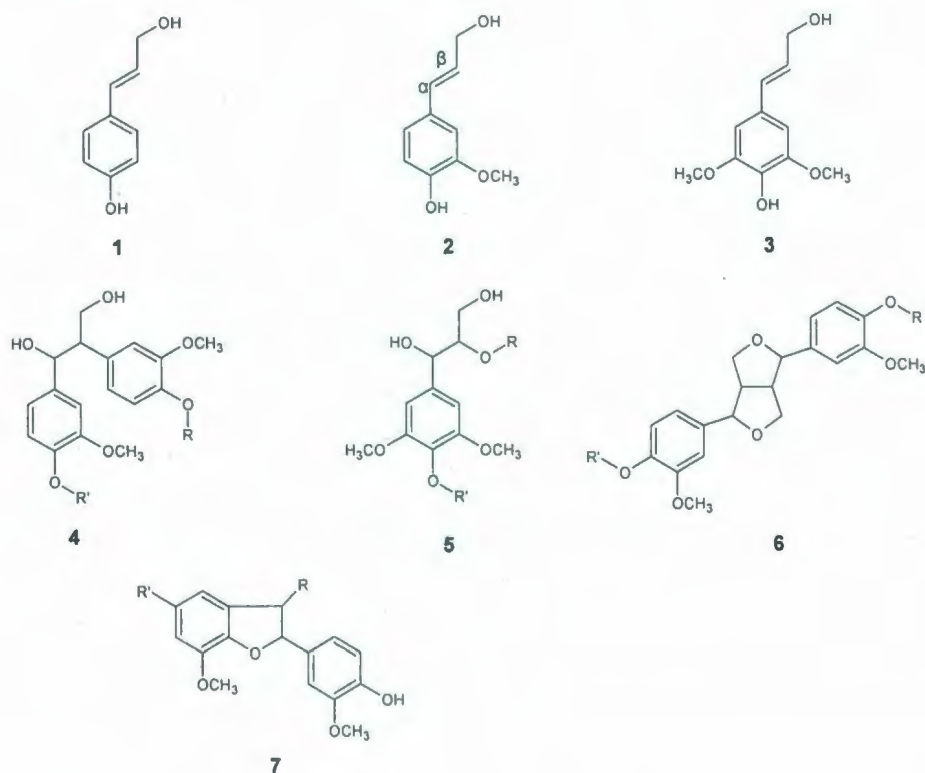


Fig. 1.7 Examples of primary lignin structures [12] and substructure subunits (1) p-coumaryl alcohol (2) guaiacyl alcohol (3) sinapyl alcohol (4) β -aryl subunit [89] (5) β -ether subunit [85] (6) β - β subunit [87] (7) β -5 subunit [22].

Lignins were studied by thermochemolysis in the presence of TMAH [85-86]. The markers resulting from thermochemolysis include 3,4-dimethoxybenzaldehyde (2), 3,4-dimethoxyacetophenone, 3,4-dimethoxybenzoic acid methyl ester (3) and 3,4-dimethoxystyrene (4) (see Fig. 1.8). Clifford et al. [85] also found methylated coniferyl derivatives resulting from thermochemolysis by the cleavage of β -O-4 bonds. Lignin

model compounds containing a β - β linkage were subjected to thermochemolysis-GC/MS in the presence of TMAH at 500 °C [87].

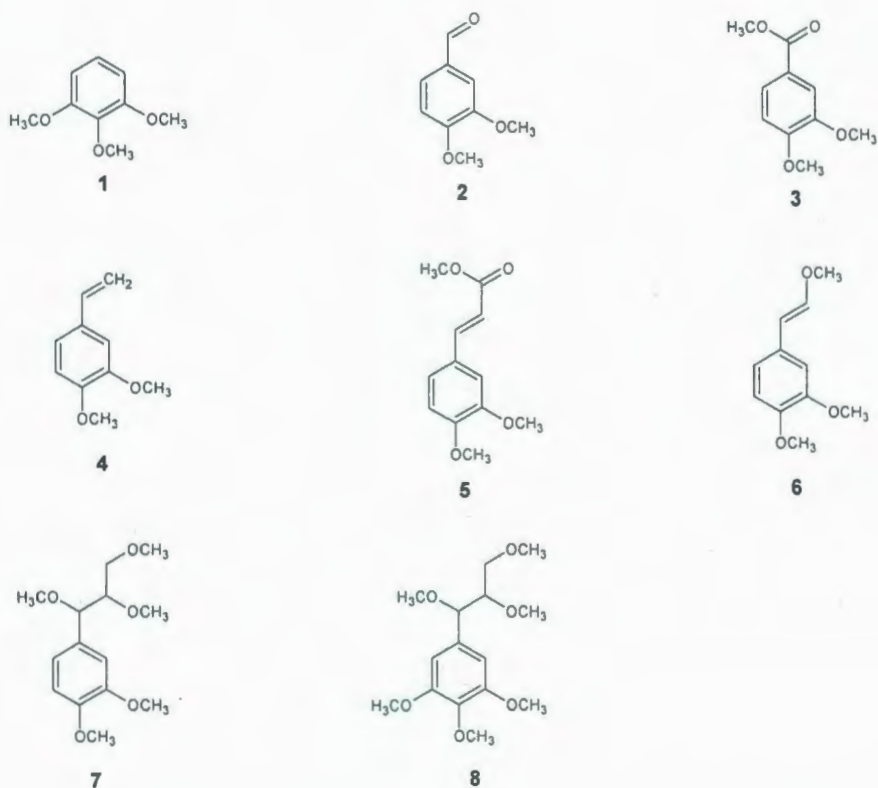


Fig. 1.8 Major lignin thermochemolysis products (1) 1,2,3 trimethoxybenzene (2) 3,4-dimethoxybenzaldehyde (3) 3,4-dimethoxybenzoic acid methyl ester (4) 3,4-dimethoxystyrene (5) 3,4-dimethoxycinnamic acid methyl ester (6) 1-(3,4-dimethoxyphenyl)-2-methoxyethene (7) 1-(3,4-dimethoxyphenyl)-1,2,3-trimethoxypropane (8) 1-(3,4,5-trimethoxyphenyl)-1,2,3-trimethoxypropane [90,91,93,94].

Pinoresinols and syringaresinol containing a β - β linkage yielded the intact O-dimethylpinoresinol and O-dimethylsyringaresinol, respectively, as the major product,

and their methylated monomers. In a study by Kuroda and Nakagawa-izumi [88] thermochemolysis of softwood lignin in the presence of TMAH at 315 °C produced two markers, 1-(3,4-dimethoxyphenyl)-2-methoxyethene (6) and 1-(3,4-dimethoxyphenyl)-1,2,3-trimethoxypropane (7), which were derived from β -aryl ether subunits. In another study, guaiacyl-syringyl mixed model compounds was subjected to thermochemolysis [89]. The products included 1-(3,4-dimethoxyphenyl)-1,2,3-trimethoxypropane (7) and 1-(3,4,5-trimethoxyphenyl)-1,2,3-trimethoxypropane (8) as β -aryl ether subunit-derived products. Products 2,3,3',4'-tetramethoxy-5-(3-methoxyprop-1-enyl)stilbene and 2,3,3',4',5'-pentamethoxy-5-(3-methoxyprop-1-enyl)stilbene are from β -5 subunit-derived products and pinoresinol dimethyl ether and syringaresinol dimethyl ether are from β - β subunit-derived products. It is believed that the lower temperatures used (< 400 °C) and sample preparation were important in obtaining such large fragment products.

¹³C-TMAH was used in thermochemolysis of lignin samples [90] where it was ascertained that the order of monohydroxyl (i.e. 3,5-dimethoxy, 4-hydroxyl in source) content of syringyl compounds in plant materials was leaves (70%) < root (75%) < bark (77%) < brown rot wood (86%) < wood (89%). Thermochemolysis-GC/MS ¹³C-TMAH was also used to differentiate lignins from condensed tannins. For example, 1,2,3-trimethoxybenzene is the thermochemolysis product of condensed tannins with a prodelphinidin B ring and also the thermochemolysis product of syringyl lignin. As well,

3,4-dimethoxy-based compounds are the thermochemolysis products of both a condensed tannin procyanidin B ring and guaiacyl lignin. These can be differentiated with thermochemolysis in the presence of ^{13}C -TMAH [91].

Other reagents have been used in thermochemolysis of lignins such as tetrabutylammonium hydroxide [92]. This reagent was able to discriminate original methyl groups from experimentally added methyl groups. An example of its usefulness was the presence of gallic derivatives originating from two different sources, i.e. lignins and condensed tannins.

1.4 Advantages and Disadvantages of Analytical Pyrolysis and Thermochemolysis

No analytical technique is without disadvantages, though some can state they have less than others. Analytical pyrolysis and thermochemolysis both deal with the decomposition of materials which are mainly polymeric structures, providing structural identification of decomposition products with the aim of characterizing and determining the source of the original material. The advantage of pyrolysis over thermochemolysis is the usage of higher temperatures which cleaves strong bonds such as C-C bonds in polymers. For example, polyethylene breaks apart into smaller hydrocarbons [93]. However, the high energy input and rate of decomposition produces a more complicated

fragmentation pattern and a larger number of pyrolysates. Thermochemolysis is a more selective approach than pyrolysis for molecules with specific bonds such as ether and ester bonds since it is carried out at a lower temperature with less extensive thermal fragmentation.

In the pyrolysis of fatty acids and lipids, a homologous series of *n*-alkenes and *n*-alkanes were produced [10], which provided less structural information about the parent fatty acid when compared to thermochemolysis which can provide intact fatty acid methylated derivatives. In thermochemolysis, fatty acids bonded within a lipid structure via ester bonds (as triacylglycerols) are converted to their free methyl esters which provide important information about the structure of original triacylglycerides. However, the breakage of ether/ester bonds could bring a disadvantage; for example, sinapyl and coniferyl acetates (with the acetyl group attached at the C γ of the lignin side-chain) were identified upon Py-GC/MS of lignocellulosic materials, whereas these acetyl linkages were cleaved during TMAH-thermochemolysis. However, there are reports that β -aryl linkages and β -ether linkages can also be cleaved in both pyrolysis [20] and thermochemolysis [85, 89]. TMAH-thermochemolysis was suggested to be suitable for the analysis of *p*-hydroxycinnamic acids linked by ether or ester bonds to cell wall components [94]. In another study, Py-GC-MS and thermochemolysis-GC-MS were used and the resulting products were compared for the analysis of waxes. A hydrocarbon pattern was observed in the pyrogram resulting from Py-GC-MS while a more

informative profile including fatty acids methyl ester and wax alcohols resulted from using thermochemolysis-TMAH [95].

Pyrolysis of resin acids at 800 °C yielded small aromatic compounds such as toluene, styrene and naphthalene [9] while thermochemolysis in the presence of TMAH produced two oxidized derivatives of methyldehydroabiatic acid, namely 7-oxo-dehydroabiatic acid methyl ester and 7-oxo-15-hydroxy-dehydroabiatic acid methyl ester [57]. In the case of diterpenoid resin acids, the advantage of thermochemolysis was the production of more informative fragments, while the disadvantage was the occurrence of secondary degradation reactions of resin acids such as base-catalyzed oxidation.

Flavanoids are an excellent example showing the preference of thermochemolysis over pyrolysis. In thermochemolysis, A-ring and B-ring structures of flavanoids can be discriminated, which leads to the characterization of a large group of flavanoids [42] while only catechol was found as the major pyrolysis fragment of catechin.

In analytical pyrolysis, the products will be underivatized with polar groups showing poor chromatographic behavior, while in thermochemolysis most functional groups are derivatized. However, the selection of thermochemolytic reagent is important since TMAH, the most common thermochemolytic reagent, is less effective in the methylation of hydroxyl groups [96]. Strong reagents such as TMAH also cause the

isomerization and/or degradation of polyunsaturated fatty acid components such as linoleic and linolenic acid [41, 97].

In both thermochemolysis and conventional pyrolysis, minimal or no sample extraction is needed and solvent interaction is minimal because it can be purged off in the early stages of analysis. It is also a very rapid profiling technique and a small amount of sample is required (< 400 µg).

1.5 Summary of Work Undertaken

Chapter 2: The application of the thermochemolysis-TMSH for the measurement of the influence of ozone exposure in white pine tree. The concentrations of small phenolic compounds and resin acids were examined. Short-term ozone fumigation affected the levels of two phenolic acids, i.e. 3-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid. Also, a substantial chromatographic difference was observed for resin acids for two different clones of the main tree species.

Chapter 3: Intact biomarkers of catechins and condensed tannins (i.e. dimers and monomers) were produced and identified using thermochemolysis-TMSH. A new thermochemolysis technique was developed which involved pre-methylation of the condensed tannin sample with

trimethylsilyl diazomethane followed by thermochemolysis-TMSH. This is the first time the intact flavonoid was observed in thermochemolysis experiments.

Chapter 4: The use of a GC injection port instead of the traditional microfurnace pyrolyzer was adapted for thermochemolysis. For this purpose a direct thermochemolysis solids injector was constructed and the unique set-up applied to the analysis of a wide range of biomaterials including sugars, fatty acids, resin acids, and flavonoids. This is the first time an intact and fully methylated saccharide was observed under thermochemolysis conditions.

Chapter 5: Another application of the direct thermochemolysis solids injector in profiling of hardwood and softwood lignins is presented. Two unique markers were produced and identified, and is a major advancement for rapidly distinguishing between hard and softwood based on the different hardwood and softwood lignin markers.

1.6 Co-authorship Statement

All experimental work was performed by the principal author except the fumigation of pine needles in Chapter 2, which was done by Mr. R.Cox, Canadian Forest Service-Atlantic Forestry Centre, Natural Resources Canada, Fredericton. The statistical analysis in Chapter 2 was performed by Dr. Helleur. In Chapter 3 Ms. S. Estevez assisted

in the initial design and in the use of TMSH as a methylating reagent. The principal author prepared the manuscripts based on Chapters 3, 4 and 5 and replied to reviewers with editing by Dr. R. Helleur. A small part of the Chapter 5 (TMAH of softwood) was performed in Dr. B. Sithole's laboratory in Paprican, FPInnovations, Montreal by the principal author.

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Chapter 2: Profiling secondary metabolites of needles of ozone-fumigated white pine seedlings by thermally-assisted hydrolysis/ methylation-GC/MS

F. Shadkami¹, R. Helleur^{1*} and R. Cox²

¹Department of Chemistry, Memorial University of Newfoundland
St. John's, NL, Canada, A1B 3X7

²Canadian Forest Service-Atlantic Forestry Centre, Natural Resources Canada
P.O Box 4000, Fredericton, NB, Canada, E3B 5P7

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Abstract

Plant secondary metabolites have an important role in the defense responses against herbivores and pathogens, and as a chemical barrier to elevated levels of harmful air pollutants. This study involves the chemical profiling of phenolic and diterpene resin acids in needles of two (ozone-tolerant and ozone-sensitive) white pine (*Pinus strobes*) clones, fumigated with different ozone levels (control, and daily events peaking at 80 and 200 ppb) for 40 days. The phenolic and resin acids were measured using thermally assisted hydrolysis and methylation (THM) gas chromatography/mass spectrometry. Short-term fumigation affected the levels of two phenolic acids, i.e., 3-hydroxybenzoic and 3,4-dihydroxybenzoic acids, in that both showed a substantial decrease in concentration with increased ozone dose. The decrease in concentration of these THM products may be caused by inhibition of the plant's shikimate biochemical pathway caused by ozone exposure. The combined occurrence of these two ozone-sensitive indicators has a role in biomonitoring of ozone levels and impact on forest productivity. In addition, chromatographic profile differences in the major diterpene resin acid compounds were observed between ozone-tolerant and ozone-sensitive clones. The resin acids anticopalic, 3-oxoanticopalic, 3 β -hydroxyanticopalic, and 3,4-cycloanticopalic acids were present in the ozone-sensitive pine; however, only anticopalic acid was present in the ozone-tolerant clone. This phenotypic variation in resin acid composition may be useful in distinguishing populations that are differentially adapted to air pollutants.

Key Words- Ozone, White pine (*Pinus strobus*), Needles, Secondary metabolites, Thermally- assisted hydrolysis/methylation , GC/MS , Phenolic acids, Diterpene resin acids

2.1 Introduction

Ground-level ozone (O₃) is recognized as the most widespread, and phytotoxic air pollutant in the northeastern part of North America (Skelly et al. 1997). Ozone is a strong oxidant, injuring plant tissues and disturbing the anatomical, biochemical, and physiological function of plants (Skäby et al. 1998; Kley et al. 1999). Also, it is known to affect carbon allocation and to cause disturbances in carbon transport within and between plant organs (Laurence et al. 1994).

Plant secondary compounds in needles often deter herbivores and interfere with infections by fungi (Beckman 2000). Thus, any changes in secondary metabolism as a result of gaseous air pollution are potentially important for the health of forests. Conifers produce oleoresin, a complex mixture of terpenoids, mainly composed of diterpene resin acids with minor amounts of monoterpenes and sesquiterpenes (Phillips and Croteau 1999). Oleoresin compounds act as feeding deterrents or stimulants to a wide variety of herbivores (Manninen 1999). Earlier studies have indicated slight O₃ effects on the concentrations of monoterpenes (Kainulainen et al. 1998) and resin acids (Kainilainen et al. 1995, 2000) in conifers, whereas O₃ increased the concentration of total phenolic

compounds (Rosemann et al. 1994; Booker et al. 1996). Another study, which examined pine (*Pinus* spp.) and spruce (*Picea* spp.) exposed to ozone and different nitrogen availability, concluded that elevated levels of O₃ do not affect the concentration of terpenoids from conifer stemwood (Manninen et al. 2002).

Lignin and other phenolic compounds in the foliage of plants may also be affected by O₃. Molecular and biochemical studies suggest that O₃ induces the synthesis of cinnamic acid derivatives via the shikimic and phenylpropanoid pathways (Rosemann et al. 1991; Eckey-Kalthenbach 1994). Phenylpropanoid derivatives that are reported to accumulate in conifer needles in response to O₃ include hydroxycinnamic acids (Kicinski et al. 1988), flavonoids (Langebartels et al. 1990) and proanthocyanidins (Jordan et al. 1991). Lignin content in needles was not significantly affected by O₃ exposure although catechin (precursor to tannins) concentration increased with increased levels (Booker et al. 1996). One study examined the concentrations of simple phenols of pine (*Pinus halepensis* Mill.) needles as bioindicators of NO, NO₂, and O₃ air pollution. Of the phenols studied, only vanillin decreased in concentration with measured increases in O₃.

The purpose of this study was to determine whether 2-yr-old white pine (*Pinus strobes*) foliage exposed to O₃ under field conditions affected the phenolic and resin acid concentrations in their needles. Comparatively little work has been done on this subject with *P. strobes*, a commercially important species in the Maritime provinces of Canada.

Pine forest productivity is predicted to be reduced by current levels of ambient O₃ in the region (Charland et al. 1995). Phenolic and resin acids were measured in the needles of clones originating from populations growing in two different regions with different air pollution levels. Profiling of the phenolic and acidic compounds was done directly on needles using a relatively new and rapid method of analysis, i.e., thermally assisted hydrolysis and methylation gas chromatography/mass spectrometry (THM-GC/MS) using trimethylsulfonium hydroxide (TMSH) as the methylating reagent. The THM-GC/MS technique has been used to measure resin acid components (Simon et al. 2001; Watts and de la Rie 2002; Scalarone et al. 2003) phenolic acids (Zhang, 1993; Zapf and Stan 1999; Yokoi et al. 2003) and lignins (Vane 2003; Klingberg et al. 2005) in complex biomaterials.

2.2 Methods and materials

2.2.1 Plant material and O₃ treatment Needles used in this investigation were from treated branches of grafted clones of two genotypes used in a clonal orchard experiment described by Charland et al. (1994). This experiment involved the ozone fumigation of 2-yr-old branches of 6-yr-old grafted white pine saplings (ramets¹) originating from Sudbury, Ontario (ozone-tolerant clone) and the Acadia Forest Experimental Station, New Brunswick (ozone-sensitive clone). Each ramet carried one replicate of all the branch treatments and received carbon-filtered air (10-15 ppb), 80 ppb,

¹ Ramet is an independent individual of a clone.

or 200 ppb ozone through separate branch chambers on randomly selected 2-yr-old branches. For this investigation, branches were fumigated daily for 40 d with O₃ [treatment profiles mimicking naturally occurring event shape maximizing at 10-15 (filtered air), 80, or 200 ppb] using a branch chamber fumigation system as outlined in Charland et al. (1995).

2.2.2 Experimental design The experiment consisted of randomly distributed clonal plots of eastern white pine within a uniform old nursery. Each plot contained at least six evenly spaced ramets. Three randomly chosen ramets (replicates) were shaded (57% shade) with shade cloth enclosures, whereas the other three were left in the open. Two-year-old branches on each ramet were randomly selected for treatment fumigations with the use of the Canadian Forest Service In-canopy branch chambers fumigation system. This system is further described in Charland et al. (1994,1995).

2.2.3 Needle samples Needles (40 from each plant) were removed from branches of each ozone treatment. Sampling was done at the same time of day to avoid diurnal fluctuations. Needles were dried at 60°C overnight, then ground to a fine powder before being stored in an airtight vial at -20°C.

2.2.4 Thermally-assisted hydrolysis methylation analysis THM-GC/MS analyses were carried out with TMSH as the preferred methylating reagent for resin acids as suggested in previous studies (Pastorova et al. 1997; Madonna et al. 2001). A small amount of ground needles (~250 µg) was placed into a pyrolysis sample cup, and 5 µL of

TMSH (0.25 M in methanol, Sigma-Aldrich) was added. The methanol was evaporated under a stream of N₂ before the sample cup was placed in the standby position of the furnace pyrolyzer. The sample/ TMSH mixture was then subjected to thermochemolysis by dropping the pyrolysis cup into the 400°C pyrolysis zone. Identification of the methylated phenolic and resin acid constituents of pine needles was based on retention times and mass spectral data of standard methylated phenolic standards and from mass spectra of methylated diterpenoid resin acids (Zinkel and Magee 1987). Relative concentrations of phenolic and resin acids are reported as TIC (total ion chromatogram) peak area of individual compounds per µg of sample analyzed. THM-GC/MS was carried out by using a vertical microfurnace pyrolyzer (Py; Frontier Lab.) interfaced to an HP5890 GC equipped with a HP 5971A MS as described in a previous paper (Estevez and Helleur 2005). A Frontier Lab UA-5 capillary column (25 m x 0.25 mm i.d; 0.25 µm thickness) was used. The instrumental settings were as follows: Py furnace, 400°C ; Py interface and GC injector, 280°C; column pressure, 10 psi He; split flow, 20 mL/min; GC oven program, 100 to 230°C at 20°C/ min, to 240°C at 1.5°C/min, to 300°C at 20 °C/min; MS interface temp. 280°C, 70 eV, full mass scan mode from 40-550 amu.

2.2.5 Statistical Analysis Mean and standard error of the methylated phenolic acid compounds **1** and **3** were calculated from resulting chromatographic mean peak area per µg of sample. Statistical analysis of mean peak areas of these compounds found in

needles exposed to ozone treatment, and shade/sun treatment for each clone-type were conducted by using the ANOVA statistical software PROC GLM in SAS (v.8.02).

2.3 Results

2.3.1 Metabolites identified by THM-GC/MS The resulting total ion chromatograms of the TMSH thermochemolysis products of fumigated needles of ozone-tolerant and ozone-sensitive white pine are shown in Fig. 2.1. Five common phenolic and four distinctive diterpenoid resin acids were identified as their permethylated derivatives (Table 2.1). The original structures of these phenolic and resin acids are shown in Fig. 2.2. Although the original purpose of this study was to examine changes in phenolic acids caused by O₃ fumigation, chromatographic profile differences of the resin acids between ozone-tolerant and ozone-sensitive clones were also observed (Fig. 2.1) showing a phenotypic variation within the two white pine clones. This distinctive resin acid profile may become useful in distinguishing tree populations that can be differentially adapted to air pollutants.

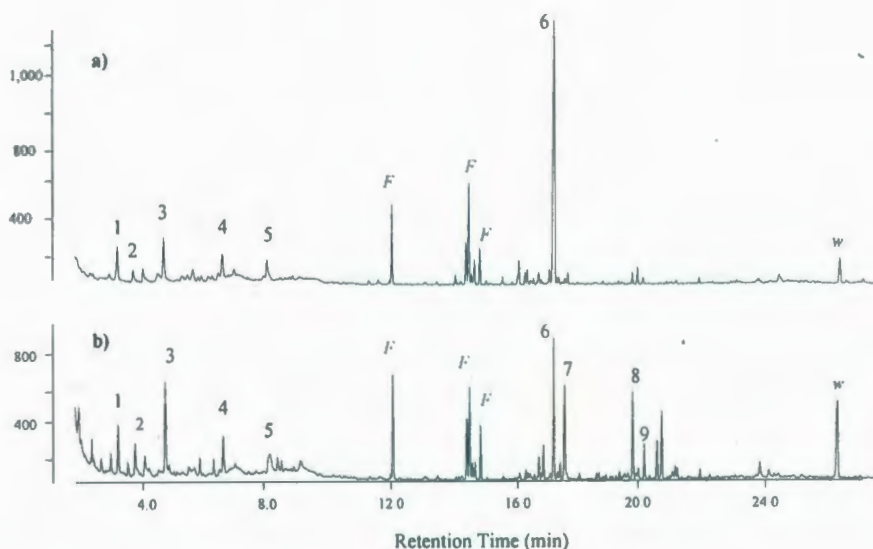


Fig. 2.1 Total ion chromatogram of the thermally-assisted methylation products of (a) needles of ozone-tolerant *P. strobus* (200 ppb O₃ treatment) and (b) needles of ozone-sensitive *P. strobus* (200 ppb O₃ treatment); The identity of the numbered peaks are listed in Table 2.1 and their non-methylated structures shown in Fig. 2.2; F- fatty acid methyl esters, w, 10-nonacosanol.

All major resin acids identified are labdane-type resin acids common in pine needles (Fig. 2.2). Anticopalic acid has been reported to be the principal resin acid in *P. strobes* needles (Zinkel and Spalding 1972) and was the most abundant resin acid in both pine clones. Three other resin acids, 3-oxoanticopalic, 3 β -hydroxyanticopalic, and 3,4-cycloanticopalic acids, were identified as the other major resin acids in needles of the ozone-sensitive pine. Conifer needles are also rich in epicuticular waxes. The major wax component of *P. strobes* as identified in Fig. 2.1 is the long chain alcohol, 10-nonacosanol.

Table 2.1 List of premethylated phenolic and resin acids identified in *P. strobus* needles using thermally assisted hydrolysis methylation GC/MS

Peak #	RT	Identity	Weight
Molecular	(min)		
1	3.45	3-Methoxybenzoic acid, methyl ester	166
2	3.82	4-Methoxybenzoic acid, methyl ester	166
3	4.58	3,4-Dimethoxybenzoic acid, methyl ester	196
4	8.74	2-Propenoic acid, 3-(4-methoxyphenyl) methyl ester	192
5	8.32	2-Propenoic acid, 3-(3,4-dimethoxyphenyl) methyl ester	222
6	17.51	Anticopalic acid, methyl ester	318
7	17.86	Cycloanticopalic acid, methyl ester	316
8	19.52	3-Oxoanticopalic acid, methyl ester	332
9	20.14	3 β -Hydroxyanticopalic acid, methyl ester	334

2.3.2 Phenolic acid levels among different treatments and white pine

populations Only 1-yr-old needles showed statistically meaningful variation in phenolic composition. 3-hydroxybenzoic and 3,4-dihydroxybenzoic acids showed significant effects ($P < 0.05$) with ozone exposure (Table 2.2). The combined results for both clones are graphically shown in Fig. 2.3. The sun/shade experiment had no statistically significant differences. These ozone-responsive phenolic compounds decrease in concentration with increased ozone dosage, by as much as 75% of the control

level in the case of 200 ppb ozone (Fig. 2.3). At the 80 ppb ozone treatment, the ozone-tolerant pine (Sudbury clone) showed a more noticeable decrease in both phenolics, whereas both clones showed a substantial decrease at high exposure (200 ppb), as illustrated in Fig. 2.4.

Table 2.2 ANOVA table for mean peak area/ μg of sample of 3-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid

Source of error	<i>df</i>	Mean Square	<i>F Value</i>	<i>p > F</i>
3-hydroxybenzoic acid				
Ozone Treatment	2	10033467.11	19.10	< 0.0001
Clone	1	5028218.45	9.57	0.0047
Sun/Shade	1	618075.12	1.18	0.2880
Rep	2	18016.85	0.03	0.9663

3,4-dihydroxybenzoic acid				
Ozone Treatment	2	4181318.00	19.58	<0.0001
Clone	1	2237615.46	10.48	0.0033
Sun/Shade	1	421018.81	1.97	0.1722
Rep	2	46563.59	0.22	0.8056

1.3.3 Resin acids and ozone treatment There was no noticeable change in resin acid concentrations in the needles of either the ozone-tolerant or ozone-sensitive white pines with O_3 fumigation. A new but unidentifiable chromatographic peak ($\text{RT}=20.83$ min, Fig. 2.1) was observed in needles of ozone-sensitive white pine fumigated at high (200 ppb) ozone levels. Its mass spectrum resembled a diterpene resin acid with a molecular weight of 332 amu. It is possible that this compound is an oxidized resin acid

metabolite of anticopalic acid formed as the result of high levels of oxidant. Further experimentation and mass spectral characterization is required to identify this product.

2.4 Discussion

This study involved the chemical profiling of phenolic and diterpene resin acids in needles of two (ozone-resistant and ozone-sensitive clone) white pine clones fumigated with different ozone levels [12-15 (control), 80 and 200 ppb] for 40 days under field conditions. The metabolites of needles were successfully measured by using a relatively new approach to "whole sample" chemical profiling, that being TMSH-GC/MS. Short-term O₃ fumigation affects the levels of two phenolic acids, i.e., 3-hydroxybenzoic and 3,4- dihydroxybenzoic acids, in that both showed a substantial decrease in concentration with increased ozone exposure; as much as a 75 % decrease in relative concentration (Figs. 2.3 and 2.4).

These two phenolic acid-THM products likely originate from the needles' photosynthetic shikimate pathway that produces significant amounts of aromatic-containing secondary metabolites (i.e., flavonoids, tannins and lignin). It is believed that ozone either inhibits the shikimate synthetic pathway, thus decreasing the amounts of 3-hydroxybenzoic acid and 3,4- hydroxybenzoic acid intermediates, or alters the pathway in preference of the synthesis of other secondary metabolite intermediates (Zhang 1993). A previous study (Kato et al. 1989) determined that the shikimate pathway was affected in cedar needles by the exposure of the plant to air pollution, including the oxidant SO₂.

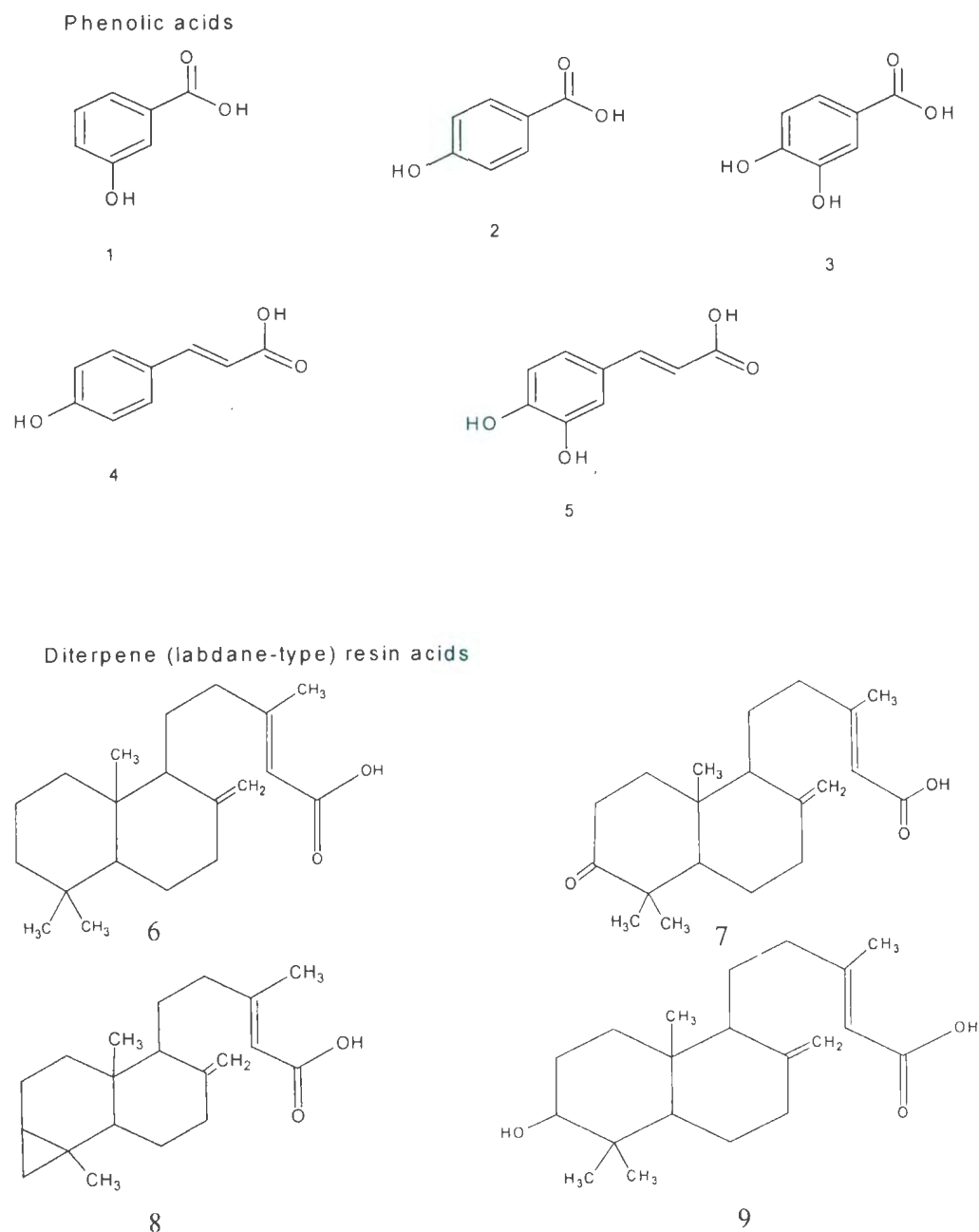


Fig. 2.2 Structures of phenolic acids and diterpene resin acids identified by THM-GC/MS in white pine needles. Compound 1 (3-hydroxybenzoic acid) and 3 (3,4-dihydroxybenzoic acid) were used as phenolic indicators of ozone exposure. The diterpene resin acids are anticopallic (6), 3-oxoanticopallic (7), 3,4-cycloanticopallic (8), and 3 β -hydroxyanticopallic acids (9).

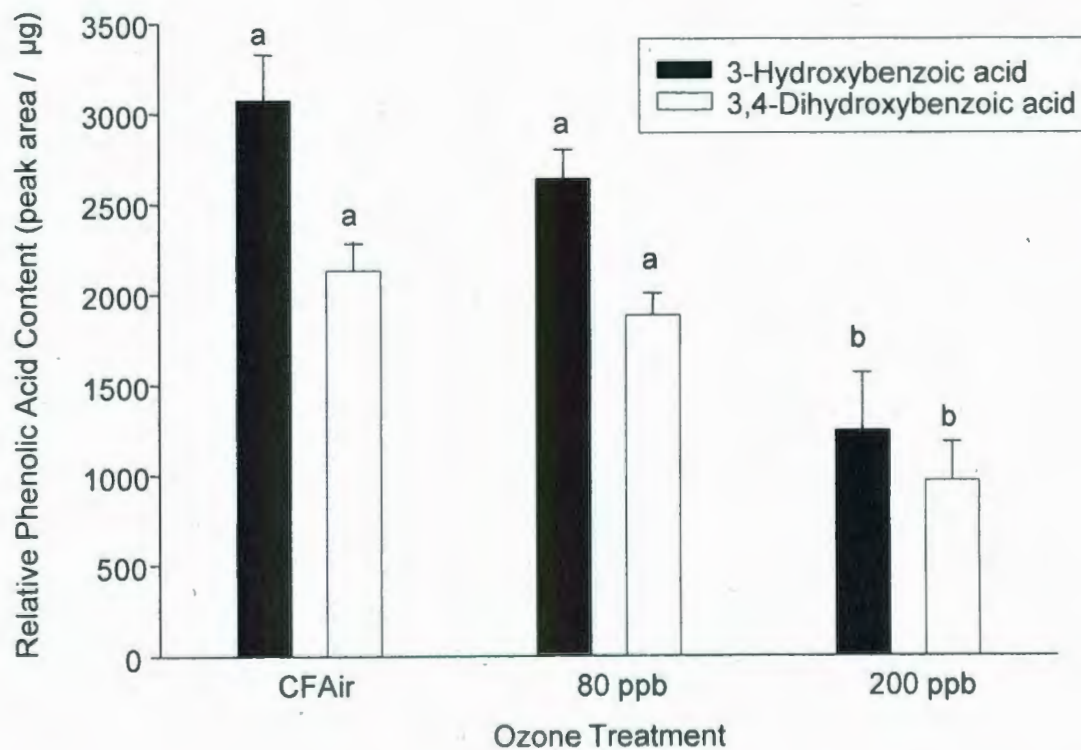


Fig. 2.3 Effect of ozone treatment on levels of phenolic acid compounds, 3-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid of all clone samples. Data values used for each sample were mean of triplicate chemical analyses. Mean values shown are from three replicate exposures, CFAir, carbon-filtered air. Letters indicate Duncan's separation of means of compound ($p < 0.001$).

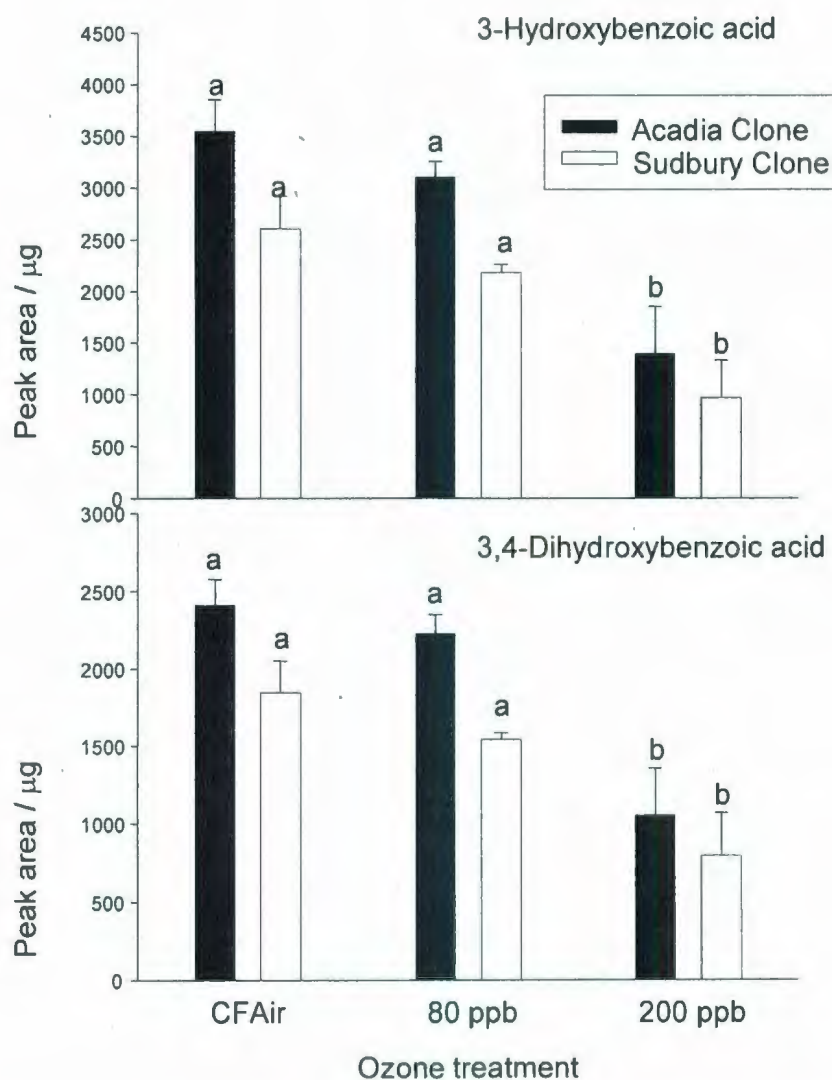


Fig. 2.4 Variation in relative content of 3-hydroxybenzoic acid and 3,4-dihydroxybenzoic acid in needles of ozone-sensitive (Acadia clone) and ozone-tolerant (Sudbury clone) *P. strobus* fumigated with ozone. Data values used for each sample were the mean of triplicate chemical analyses. Mean values shown are from three replicate exposures ± 1 SE. CFAir, carbon-filtered air. Letters indicate Duncan's separation of means by clone, ($P < 0.001$)

Our study has shown the presence of two ozone- sensitive chemical markers. Monitoring of these two phenolics may play a future role in biomonitoring of ozone levels and its impact on forest productivity. Our experimental approach to using rapid THM-GC/MS analysis is compatible with rapid chemical profiling of biomaterials as it involves little sample preparation, uses no extraction solvents, and has the advantage of utilizing the whole sample which allows for other biomarkers to be profiled simultaneously (i.e., fatty acids, terpenes, waxes, phenols, etc.).

Compositional differences in the major diterpene resin acids were observed between the needles of ozone-tolerant and of ozone-sensitive pine populations. The four major resin acids, anticopalic, 3-oxoanticopalic, 3 β -hydroxyanticopalic and 3,4-cycloanticopalic acids, were present in the ozone-sensitive pine, whereas only anticopalic acid was present in the ozone-tolerant (Fig. 2.1). This phenotypic variation in resin acids within these white pines may be useful in distinguishing populations that may be differentially adapted to air pollutants.

THM-GC/MS data from the white pine needles fumigated at high ozone exposure (200 ppb) indicate the presence of an oxidized diterpene resin acid product. Future research will be undertaken to identify this and possibly other O₃ oxidation products as additional chemical markers to ozone exposed trees.

2.6 Acknowledgment

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Chapter 3: Analysis of Catechins and Condensed Tannins by Thermally-Assisted Hydrolysis/Methylation-GC/MS and by a Novel Two-step Methylation

Farzad Shadkami, Sandra Estevez and Robert Helleur*

Analytical Chemistry Group, Department of Chemistry, Memorial University of Newfoundland,

St. John's, Nfld, CANADA, A1B 3X7

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Abstract

The objective of this study was to observe high molecular weight markers of catechins and condensed tannins by thermally-assisted hydrolysis/methylation (THM)-GC/MS. Techniques for formation of intact methylated flavanols of catechins using THM in the presence of trimethylsulfonium hydroxide (TMSH), and of a dimer marker of condensed tannins using a novel two-step methylation technique are presented. The two-step methylation procedure involves pre-methylation of the sample with trimethylsilyl diazomethane (TMS-diazomethane) followed by THM. The dimer marker, a methylated product containing a C-C linkage between adjacent flavanol units, has a molecular weight of 540 amu. Intact methylated flavanols of catechins were also successfully observed as high molecular weight compounds including partially methylated catechin and epicatechin (3-Flavanol, 3',4',5,7-tetramethoxy, cis/trans; $m/z = 346$), epigallocatechin and gallocatechin (3-Flavanol, 3',4',5,5',7-pentamethoxy, cis/trans; $m/z = 376$). These techniques were successfully applied to the analysis of a series of condensed tannins isolated from plants, and catechins and other phenolics present in (hot water) extracts of tea leaves. In green tea, the major catechins were identified as epicatechin and epigallocatechin along with flavonols and tannin dimers.

Keywords: thermally assisted hydrolysis and methylation (THM); tetramethylammonium hydroxide (TMAH); trimethylsulfonium hydroxide (TMSH); trimethylsilyl diazomethane; gas chromatography/mass spectrometry; condensed tannins.

3.1 Introduction

Thermally-assisted hydrolysis/methylation (THM) or thermochemolysis has previously been used for the analysis of natural plant matter [1,2,3,4]. In these studies the organic bases, tetramethylammonium hydroxide (TMAH) and trimethylsulfonium hydroxide (TMSH), were applied off-line [3] or *in situ* [1,2,4] using a pyrolysis device attached to a gas chromatograph to analyze, in particular, phenolic and carboxylic acid units in lignins, lipids, and resin acids. Since methylated products are more volatile molecules than their unmethylated precursors, they are suitable for analysis by gas chromatography/mass spectrometry. THM is complementary to conventional or direct pyrolysis and provides more definitive structural information of the original composition of the sample. In direct pyrolysis, large molecules are extensively fragmented into smaller units in the absence of oxygen at temperatures from 600°C to 800°C.

Condensed tannins are the second most abundant family of natural phenolic compounds, after lignins. They are secondary metabolites primarily found in the bark, root, and leaves of most plant species but also in seeds and fruits. They have the ability

to bind to proteins and carbohydrates, which reduces digestibility of tannin rich food for herbivores. Catechins and condensed tannins are important for human health acting as antioxidants [5,6]. They are also important for the health of plants which contain them, acting as phytoalexins [7].

Catechins, including catechin, epicatechin, epigallocatechin and gallocatechin, belong to a family known as flavan-3-ols (or 3-flavanols). These catechins are also the main monomeric units present in condensed tannins (Fig. 3.1). Condensed tannins can vary in the number of hydroxyl groups in the B-ring. Procyanidin (PC) condensed tannin contains two hydroxyl groups (e.g. catechin and epicatechin monomers), while prodelphinidins (PD) condensed tannin contain three hydroxyl groups (e.g. epigallocatechin and gallocatechin monomers). Condensed tannins are flavanol units that can be either singly linked at the 4-6 or 4-8 positions (B-type), or doubly linked at the 4-8 position and via an oxygen between position 2-7 (A-type). The B-type condensed tannins are more common in food products especially in cocoa, grapes, and apples while the A-type condensed tannins are more rare and are present in peanuts and cinnamon .

The chemistry of catechins has been extensively investigated since the 1970s. Since these compounds will only be subjected to the basic and thermal conditions (i.e. THM conditions) in this study, a brief summary of their chemical behavior under these conditions will be discussed. It is well-known that catechin and epicatechin can

interconvert between one another through epimerization under basic conditions [8,9,10] or as a result of photo-isomerization [11]. Several compounds were isolated in a base-catalyzed reaction of catechin and epicatechin at high pH and different temperatures. For example, catechinic acid was formed in reactions at a high pH and 100°C. Also, the catechinic acid stereoisomer was obtained when catechin and epicatechin were subjected to pH 12 and 40°C. In addition, at a high pH and 25°C, catechin and epicatechin epimerized to one another. Analysis was performed by methylation followed by NMR and mass spectrometry [12,13].

Characterization of condensed tannins in plant sources is mainly by NMR [12,13,14,15] but a relatively pure isolate is needed which is a disadvantage for mixtures. Among chromatographic techniques, normal or reversed phase liquid chromatography mass spectrometry (LC-MS) equipped with ESI and APCI ion sources [16,17] and size-exclusion chromatography [18] are the principal methods used for the analysis of catechins and condensed tannins. Of the more rapid techniques of analysis, fast atomic bombardment mass spectrometry [12] and matrix assisted laser desorption ionization (MALDI) with a mass spectrometer as a detector [19,20] have been used to determine the molecular weight and the degree of polymerization of oligomeric and polymeric condensed tannins. Fast atomic bombardment rarely produces informative fragmentations and has a poor sensitivity. The analysis of small molecules (<1000 Da) by MALDI is always problematic due to high abundance of matrix ions.

Thermally-assisted hydrolysis/methylation gas chromatography mass spectrometry has been employed for *in situ* analysis of condensed tannins. In this technique, no sample extraction is needed and solvent interaction is minimal because it can be purged off in the early stages of the THM process. It is also very rapid and small amounts of sample are used (~500 µg). The THM-GC/MS studies using TMAH [14,21,22,23] resulted in small fragments of the catechins and condensed tannins. All three studies reported A-ring THM products of 1,3,5-trimethoxybenzene and 2,4,6-trimethoxytoluene. However, only Nierop et al. [14] and Galletti and Bocchnini [22] found 2-ethyl-1,3,5-trimethoxybenzene as an A-ring THM product. It should be noted that procyanidin and prodelphinidin have the same A-ring structure, therefore, A-ring markers can not differentiate procyanidin from prodelphinidin. Garnier et al. [21] found significant amounts of 3,4-dimethoxybenzoic acid methyl ester and 3,4-dimethoxystyrene as B-ring products of catechin and epicatechin while Nierope et al. [14] and Galletti and Bocchnini [22] reported small amount of 1,2-dimethoxybenzene as the main B-ring products of catechin and epicatechin. For the analysis of epigallocatechin units, both Garnier et al. [21] and Nierop et al. [14] reported 1,2,3-trimethoxybenzene, 3,4,5-trimethoxytoluene and 3,4,5-trimethoxybenzaldehyde. As well, Nierop et al. [14] assigned 1,2,3,5-tetramethoxybenzene as a prodelphinidin marker and 1,2,4-trimethoxybenzene as a procyanidin marker which was not reported by the other two studies. They also suggested 1,3,5-trimethoxybenzene as a marker of tannins in general.

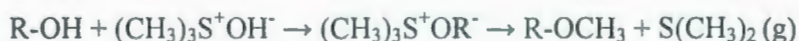
The presence of condensed tannins in real samples, which include grape skin, grape seeds, wine, and archaeological wine residues, were also investigated [21,23]. Garnier et al. [21] discussed the formation mechanism of the THM products and assigned more than one origin for the THM products. These assignments suggest that these products can originate from different classes of the compounds.

The results of above studies have their limitations. There is no intact 3-flavanol, no products from the C-ring of 3-flavanol, and no marker for the presence of the C-C linkage between adjacent flavanol units. In some cases, some of the markers cannot be differentiated from markers of other classes of compounds. For example, the presence of the B-ring product of 3,4-dimethoxybenzoic acid methyl ester in wine can be associated with a common phenolic acid in trees [4] and another flavonoid class, anthocyanidins (peonidin) [21]. Also, some lignin and sugar markers are similar to the proposed markers of catechins and condensed tannins [14,21].

The conditions of THM are also problematic. Most of the above studies use temperatures above 450°C, in fact, some as high as 600°C [14,22]. It is very likely that this high temperature results in excessive thermal fragmentation. For example, that is why 1,2-dihydroxybenzene (1,2-dimethoxybenzene in THM) is observed under both THM [14,22] and conventional pyrolysis [24]. In fact, the high temperature used for

quaternary ammonium salts decomposition [25,26] favors excessive decomposition and unwanted reactions rather than a selective, base-catalyzed hydrolysis and methylation.

Similar to TMAH, TMSH (trimethylsulfonium hydroxide) is an organic base which has been used as a methylating reagent in organic synthesis [27], requiring lower THM temperatures [28,29]. In THM using TMSH [27], similar to that of TMAH [30], phenolics (or carboxylic acids) undergo the following reaction:



This chapter will describe techniques for the formation and identification of unique chemical markers of catechins and dimers from condensed tannins using THM-GC-MS and the two-step methylation technique. Intact catechin derivatives were successfully observed by THM using TMSH. Dimer-related products were only observed when the two-step methylation procedure was undertaken. This involved pre-methylation followed by THM in the presence of TMSH. The emphasis of this chapter is on new techniques using TMSH and TMS-diazomethane (trimethylsilyl diazomethane) as main derivatization reagents.

3.2 Experimental

3.2.1. Samples and reagents

Catechin, epicatechin and epigallocatechin, used as model compounds (Fig. 3.1) of condensed-tannin monomeric units, were purchased from Sigma (St. Louis, MO, USA) while the B1 dimer condensed tannin (epicatechin-(4 β -8)-catechin) was purchased from Chromadex (Irvine, CA, USA). All reagents (Fig. 3.2), i.e., methanolic solutions of TMAH (25% w/w), TMSH (0.25 M, ~2.5% w/w) and trimethylsilyl diazomethane in dimethylether (2 M) were also purchased from Sigma. The reagents were used as is except for the THM temperature optimization (Section 3.3.2) where the TMAH solution was diluted with methanol to a concentration of 2.5%. Plant leaf tannin extracts were kindly donated by Dr. Preston, the Pacific Forestry Centre, Victoria, BC [14]. The green tea leaves were President's Choice brand (Dominion stores). For the preparation of green tea extract, the contents of one tea bag were placed into a vial containing 10 ml of water capped and heated for 10 min at 80°C. The solution was not allowed to be heated over 80°C as another study [31] found that epimerization of catechin occurred at higher temperatures. A 20 μ l aliquot of tea extract was used during THM experiments.

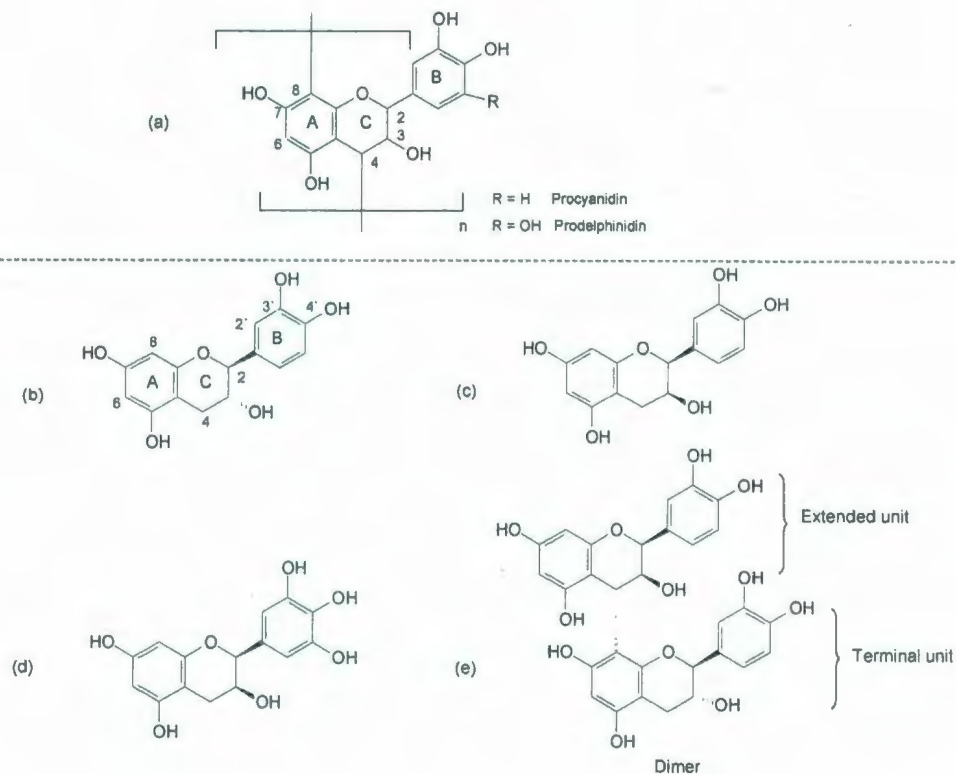


Fig. 3.1 Chemical structures of model compounds (a) a condensed tannin ($n = 2$ to 12) (b) catechin, (c) epicatechin, (d) epigallocatechin and (e) epicatechin-(4 β -8)-catechin (B1).

3.2.2 Pyrolyzer-gas chromatography-mass spectrometry

The following procedure and instrumentation were used in all gas chromatography-mass spectrometry (GC-MS) analyses. In order to introduce the sample, the sample cup (a small u-shape stainless steel sample cup: Frontier Lab), was first placed

in the waiting position of the vertical furnace pyrolyzer. The sample/reagent mixture was then subjected to heat by dropping the pyrolysis cup into the pyrolysis furnace. In a few runs, experiments were also repeated using a flow-through sample cup as described by Hosaka [32]; nevertheless, no significant differences were observed. The cup was introduced to a Frontier Lab vertical micro-furnace pyrolyzer interfaced to an HP5890 GC equipped with an HP5971A MS as described in a previous paper [29]. A ZB-5HT Inferno capillary column (15 m, 0.32 mm id and 0.10 μm film thickness) with maximum temperature limit of 400/430°C was used. The pyrolysis interface and GC injector were set at 350°C and the MS interface was at 300°C. The column pressure was 5 psi and the split flow 20 ml min⁻¹. Oven temperature was 100°C for 5 min then 20°C min⁻¹ to 375°C, and held for 5 min. The mass spectra scan range was from 60 to 650 amu. Identification of methylated products was found using the NIST library and from spectra found from literature data.

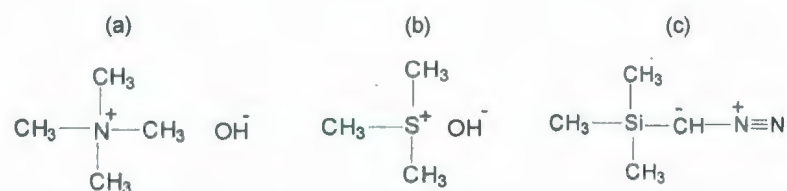


Fig. 3.2 Chemical structures of reagents (a) tetramethylammonium hydroxide (TMAH), (b) trimethylsulfonium hydroxide and (c) trimethylsilyl diazomethane (TMS-diazomethane).

3.2.3 THM procedures

In all THM procedures 500 μg of standards and tannin samples were used. For the tea leaf a 20 μl aliquot of tea extract was added into a small vial, the vial was placed in a hot metallic container, purged with N_2 and dried. The sample was added into 35 μl of methanolic TMAH or TMSH and sonicated. A 5 μl aliquot of the solution was placed into a sample cup. An effective concentration for TMSH was assigned at the original TMSH concentration i.e. 0.25 M. The hydroxyl groups (catechin)/THM reagents mole ratio produced the best result at about 1/1.2 mole ratio, which is equivalent to using 500 μg catechin for 35 μl TMSH.

3.2.4 Methylation using TMS-diazomethane

For methylation at r.t., 500 µg of standard/sample was dissolved in 30 µl of methanol, then 30 µl of TMS-diazomethane was added to the vial. The sample was sonicated for 30 min. Optimized conditions for hydroxyl groups (catechin)/TMS-diazomethane were at a mole ratio of 1/1.5. A 5 µl aliquot of the final solution was placed in a pyrolysis sample cup. For the amount of tea leaf sample used, see Section 3.2.3.

3.2.5 Two-step methylation technique

In two-step methylation (i.e. TMS-diazomethane followed by THM) the TMS-diazomethane procedure (Section 3.2.4) was followed except that the solution was evaporated under a stream of N₂. A 40 µl of 2.5% TMSH or 0.25% TMAH was added to the dried sample. A 5 µl aliquot of this solution was used for analysis.

3.2.6 Off-line two-step methylation

The off-line pyrolysis apparatus was illustrated in the previous study [33]. A 5 µl aliquot of the solution (Section 3.2.5) was placed on a small plug of quartz wool in the quartz tube.

Table 3.1 Chemical assignment of major chromatographic peaks

Peak # ^a	MW	RT (min)	Product ^b
1	226	7.5	3,4,5-trimethoxybenzoic acid methyl ester
2	168	8.9	1,3,5-trimethoxybenzene
3	182	9.7	2,4,6-trimethoxytoluene
4	196	10.1	2-ethyl-1,3,5-trimethoxybenzene
5	360	19.0	Flavan, 3,3',4',5,7-pentamethoxy, trans (fully methylated form of catechin)
6	360	19.0	Flavan, 3,3',4',5,7-pentamethoxy, cis (fully methylated form of epicatechin)
7	390	19.3	Flavan, 3,3',4',5,5',7-hexamethoxy, cis (fully methylated form of epigallocatechin)
8	346	19.5	3-Flavanol, 3',4',5,7-tetramethoxy, trans (partially methylated form of catechin)
9	360	19.6	1-(3,4-dimethoxyphenyl)-3-(2,4,6-trimethoxyphenyl)-1-propen-2-ol
10	346	19.7	3-Flavanol, 3',4',5,7-tetramethoxy, cis (partially methylated form of epicatechin)
11	376	19.8	3-Flavanol, 3',4',5,5',7-pentamethoxy, trans (partially methylated form of gallocatechin)
12	376	19.9	3-Flavanol, 3',4',5,5',7-pentamethoxy, cis (partially methylated form of epigallocatechin)
13	328	21.0	3-Flavonol, 3',5,7-trimethoxy (partially methylated form of kaempferol)
14	358	21.2	3-Flavonol, 3',4',5,7-tetramethoxy (partially methylated form of quercetin)
15	540	22.0	Flavan, 3,3',4-(3-methyl,2,4,6-trimethoxyphenyl)-4',5,7-pentamethoxy, cis/trans
16	540	22.5	Isomer of #15
17	570	23.0	Flavan, 3,3',4-(3-methyl,2,4,6-trimethoxyphenyl)-4',5,5',7-hexamethoxy, cis/trans

a. as shown in chromatograms.

b. some structures shown in Fig. 3.6.

The quartz tube was positioned inside the coil of a pyroprobe (Chemical Data Systems). The coil was heated inside an inert nitrogen atmosphere of the water condenser part of a micro-sublimator (Kontes Glassware). The condensed pyrolysate, which formed inside the quartz tube, was washed off with 10 μ l of methanol.

3.2.7 Direct Chemical Ionization (DCI) Mass Spectrometry

In this experiment, a Waters Micromass GCT Premier time-of-flight mass spectrometer equipped with probe inlet was used. Chemical ionization plasma was produced using methane as an ionization gas. The Direct Chemical Ionization or Desorption Chemical Ionization (DCI) probe was slowly ramp-heated by passing a current of 0 to 1.6 A in 10 min. The ion source temperature was set at 150°C.

For DCI-MS, 1 μ l of the solution product from the off-line two-step methylation (Section 3.2.6.) was added directly to the DCI wire and the methanol evaporated. The sample was heated and vaporized rapidly by passing an electric current through the wire. The mass analyzer scans the compounds which are released as a result of desorption from the metal wire and subsequently chemically ionized in the CI plasma.

3.3 Results and Discussion

3.3.1 Preliminary THM experiments

A preliminary investigation of THM of catechin was undertaken using similar conditions to those found in the literature (25% TMAH at 600°C). The results for catechin yielded almost the same compounds (Fig. 3.3, Table 3.1) which were reported by Nierop et al. [14], Garnier et al. [21], and Galletti and Bocchini [22] in spite of using different pyrolysers. These compounds include 1,3,5-trimethoxybenzene, 2,4,6-trimethoxytoluene and 2-ethyl-1,3,5-trimethoxybenzene which was reported [14,23]. Further experiments were not able to differentiate between catechin and epicatechin and some of the products were also common to epigallocatechin. Furthermore, many of our THM products are A-ring derived and the configuration of the A-ring is the same for all condensed tannins. Moreover, as previously discussed [14,22], for complex mixtures of polyphenolics many markers of catechins/condensed tannins are similar to those of lignin, and, also similar to phenolic products produced from THM of monosaccharides [34].

The focus of this study is to establish THM conditions to produce higher molecular weight markers and lessen the degradation of catechins. Very promising results (Fig. 3.4(a)) were obtained when catechin was subjected to THM in the presence

of TMSH. As it is seen, in contrast to TMAH, the markers from THM of catechin in presence of TMSH are higher molecular weight compounds which uniquely represent a catechin molecule (discussed in more detail in Section 3.3.3).

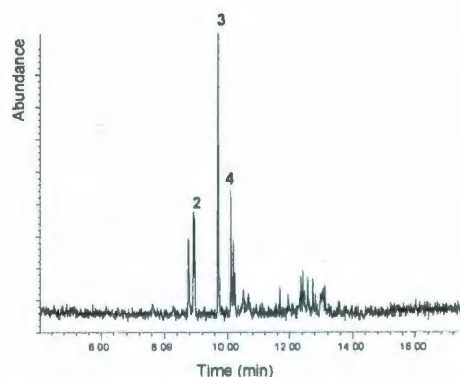


Fig. 3.3 Total ion chromatogram GC/MS obtained from THM (25% w/w TMAH, 600°C) of catechin.

3.3.2 Optimization of THM temperature using TMSH vs TMAH

A thorough investigation of the choice of THM reagent and the selection of optimization temperature was carried out. TMSH was chosen because, from previous studies, it was a less harsh THM reagent than TMAH [28,29]. Figure 3.5 shows the relationships between THM temperature and the relative total production of small phenolics (2, 3 and 4; Fig. 3.6.) and high molecular weight markers (8 and 6; Fig. 3.6)

produced from THM of catechin using TMAH or TMSH. Choice of temperature was critical for the production of unique high molecular weight products using TMSH where a low temperature of 250°C produced the best yield. Lower temperatures were not investigated due to condensation of products in pyrolysis zone.

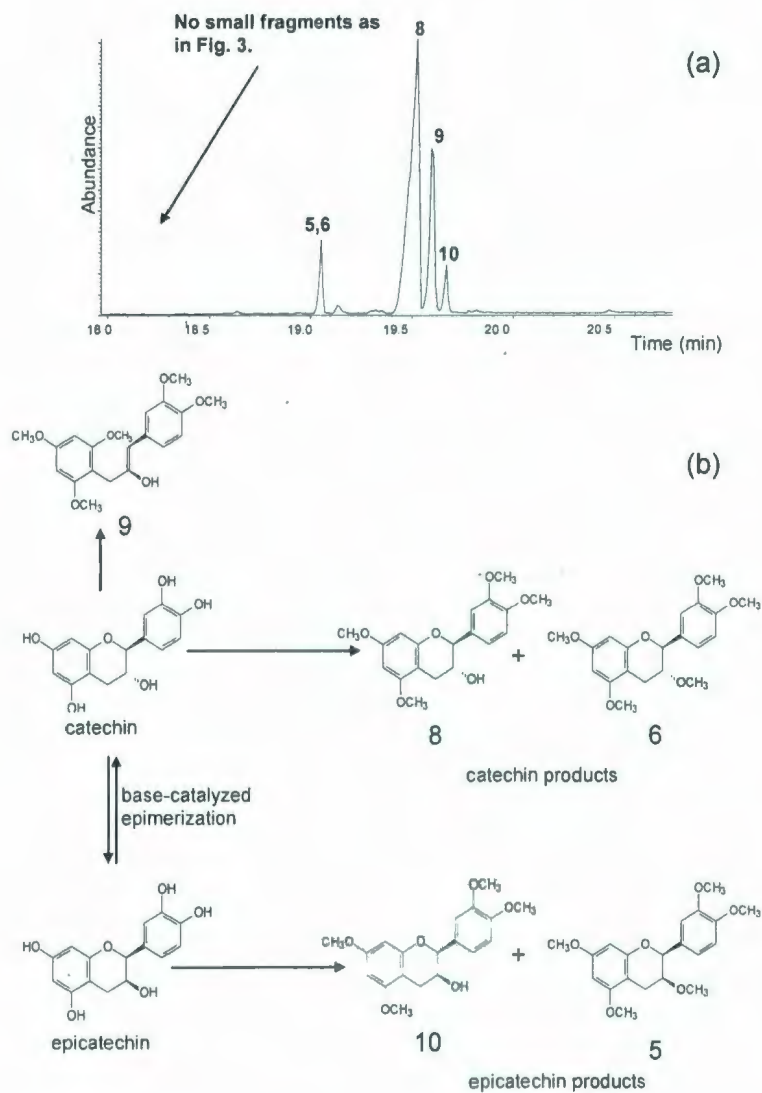


Fig. 3.4 (a) Partial total ion chromatograms obtained from THM (in presence of TMSH) of catechin at 250°C, (b) base-catalyzed epimerization and THM (in presence of TMSH) products of catechin and epicatechin.

3.3.3 THM-GC-MS of catechin and epicatechin

THM-GC-MS of catechin and epicatechin in the presence of TMSH were performed. In Fig. 3.4(a), the THM of catechin, the chromatogram shows fully methylated catechin and epicatechin (**5** and **6**) and partially methylated catechin and its isomer epicatechin (**8** and **10**). Compound **9** is believed to be the result of C-ring opening of catechin. Although numerous products were obtained from the THM of catechin, by far the largest peak (**8**), partially methylated catechin, makes this a useful marker.

The methylated epicatechin (**10**) is the result of epimerization of catechin under basic condition as shown in Fig 3.4(b). Careful experimental procedure could not lessen this inherent epimerization.

Another remarkable result of the THM-GC-MS of catechin (trans epimer) and epicatechin (cis epimer) was the large retention time difference between partially methylated catechin (**8**) and epicatechin (**10**). Product **10** was the dominant peak in the chromatogram resulting from the THM of epicatechin, thus, this chapter has shown that one can discriminate between epicatechin and catechin using the THM approach of analysis.

The mass spectra of partially methylated and fully methylated catechin and epicatechin are given in Fig. 3.7 [35].

3.3.4 THM-GC-MS of epigallocatechin

The products observed in the chromatogram (Fig. 3.8) from the THM of epigallocatechin in the presence of TMSH were fully methylated epigallocatechin (**7**; Fig. 3.6) and partially methylated epigallocatechin (**12**; Fig. 3.6). Surprisingly, epimerization did not occur in THM for epigallocatechin under basic conditions, possibly because of the greater number of hydroxyl groups in the epigallocatechin's B-ring. The mass spectra of partially methylated and fully methylated forms of epigallocatechin can be found in Fig. 3.7.

3.3.5 Methylation of catechin and epicatechin using TMS-diazomethane

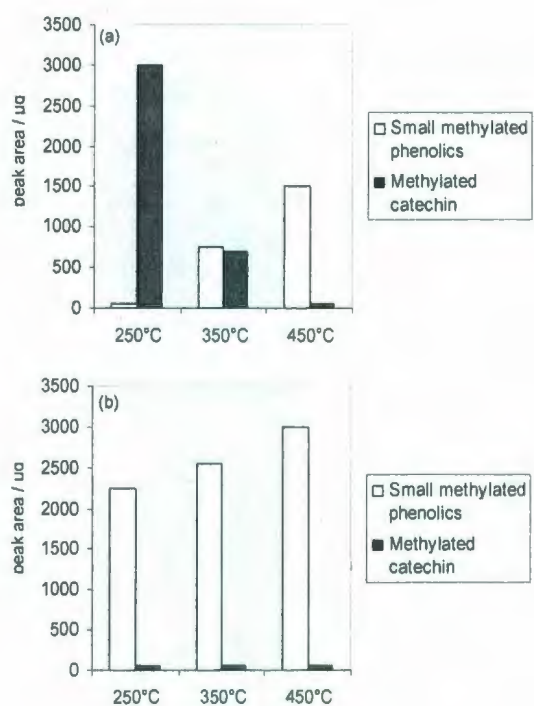


Fig. 3.5 Relationships between THM temperature and relative contents of small phenolics and catechins produced using (a) TMSH and (b) TMAH.

TMS-diazomethane was used to further verify the identity of fully methylated and partially methylated catechin and epicatechin, and also to thermally stabilize the structure

of condensed tannins (see Section 3.3.6). TMS-diazomethane is an easy to use reagent; it is safer than its counterpart methylating reagent, diazomethane, and it only requires 30 minutes of reaction time. No interfering artifacts in analysis with GC-MS were observed [36].

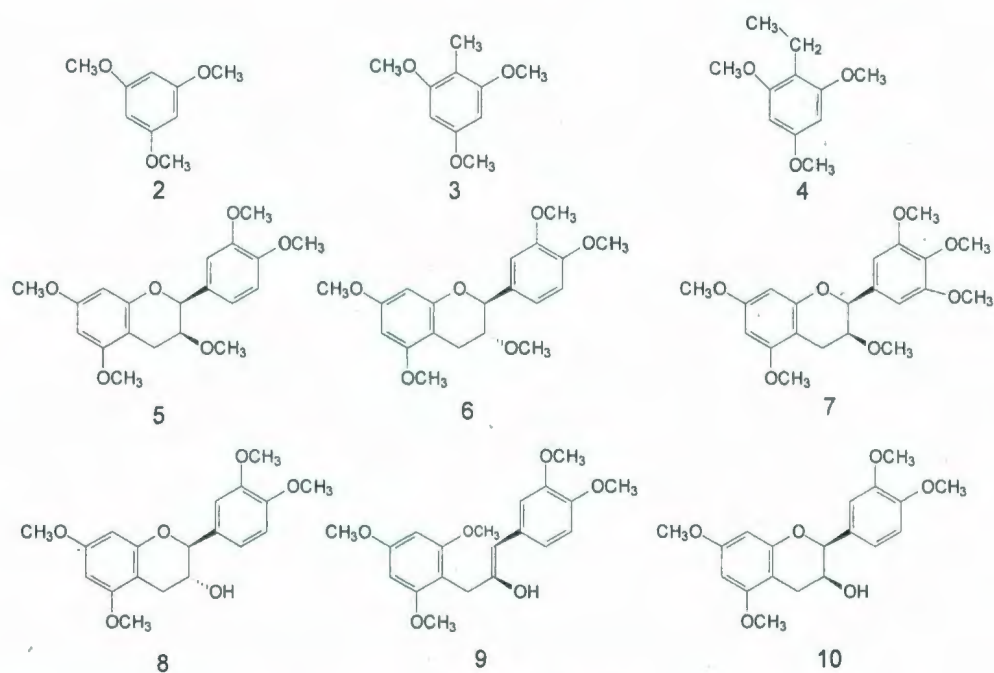


Fig. 3.6 Structures of THM products identified by THM-GC/MS as listed in Table 1.

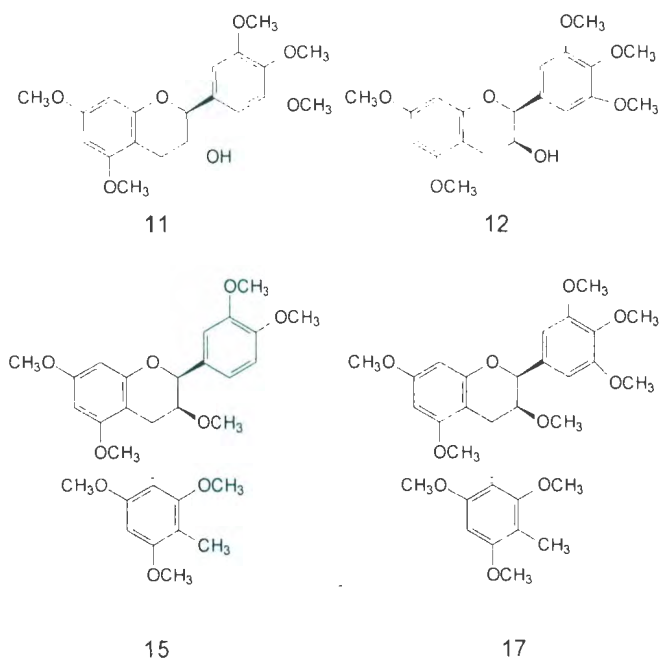


Fig. 3.6 (continued)

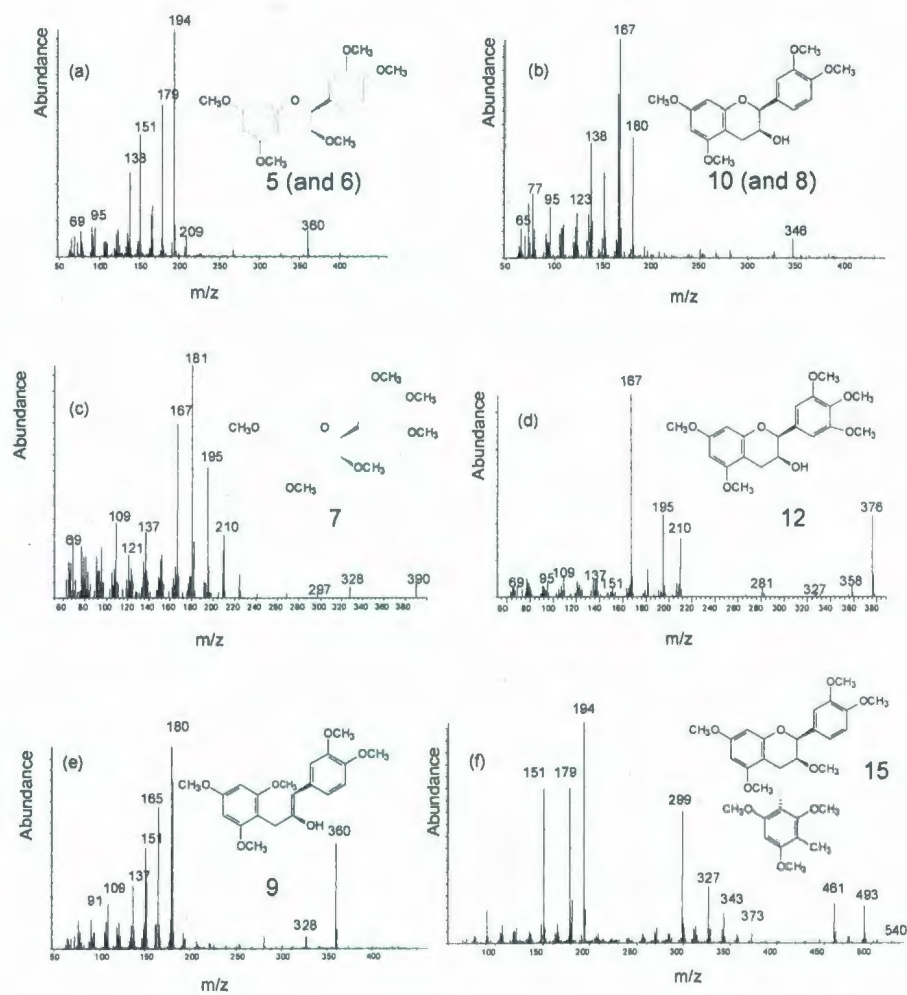


Fig. 3.7 EI mass spectra of methylated products (a) 5, (b) 10, (c) 7, (d) 12, (e) 9 and (f) dimer marker 15.

Figure 3.9(a) shows gas chromatograms related to methylation products of catechin (Fig. 3.9a(i)) and epicatechin (Fig. 3.9a(ii)) using TMS-diazomethane wherein the partially methylated catechin (**8**) and epicatechin (**10**) are the main products as shown in the reaction equation of Fig. 3.9(b). Smaller amounts of fully methylated catechin (**6**) and epicatechin (**5**) were also observed both at the same retention time (Table 3.1). However, compounds **8** and **10** have very different retention times. It is likely that the presence of an alcoholic hydroxyl group which can hydrogen bond is the reason for the chromatographic separation of these two isomers. In compounds **8** and **10** the phenolic groups are methylated while the less acidic alcoholic hydroxyl group remains unreacted.

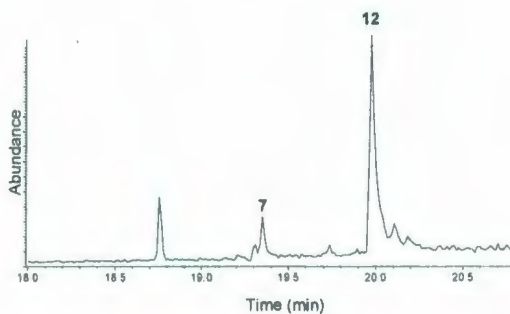


Fig. 3.8 Partial total ion chromatogram obtained from THM (in presence of TMSH) of epigallocatechin at 250°C.

3.3.6 Analysis of Tannin dimer: THM only vs two-step methylation

The chromatogram resulting from THM (TMSH) of B1 dimer (epicatechin-(4 β -8)-catechin) is presented in Fig. 3.10a(i). The largest peak is related to our catechin marker (8) as the result of the known base-catalyzed reaction of condensed tannins whereby the terminal unit, in this case catechin, retains its chemical structure [15]. Verification that no monomers were present in the dimer standard was carried out using TMS-diazomethane. It may be concluded that, under THM conditions, the interflavanoid bond (Fig. 3.1(a)) is broken but with low yield and likely only for the terminal unit.

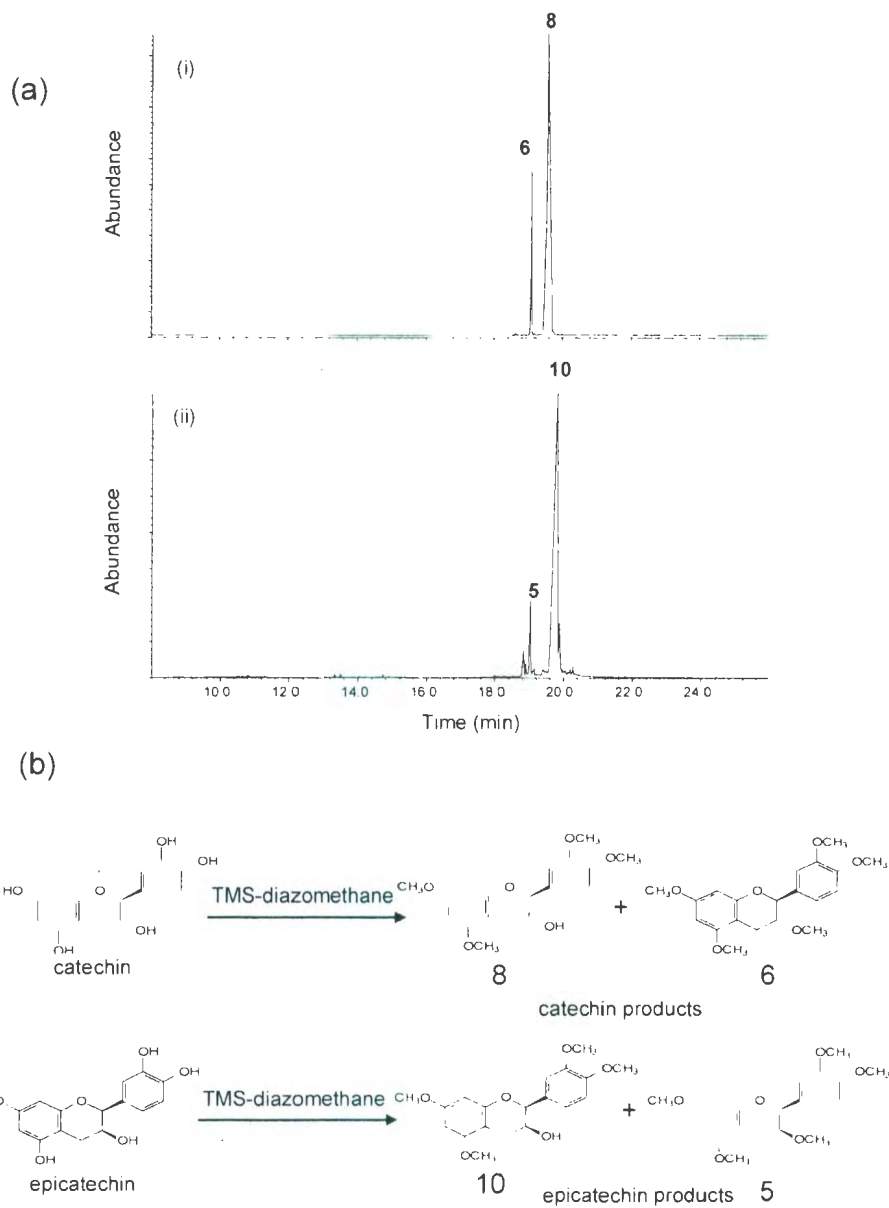


Fig. 3.9 (a) Partial total ion GC/MS chromatograms obtained by methylation using TMS-diazomethane of (i) catechin and (ii) epicatechin, (b) methylation products of catechin and epicatechin using TMS-diazomethane.

To further explore the possibility for the obtaining a marker for tannin dimer (i.e. a product containing a C-C interflavanoid linkage) a two-step methylation technique was developed. This involves pre-methylation of the analyte with TMS-diazomethane followed by THM-GC-MS as described in the experimental section.

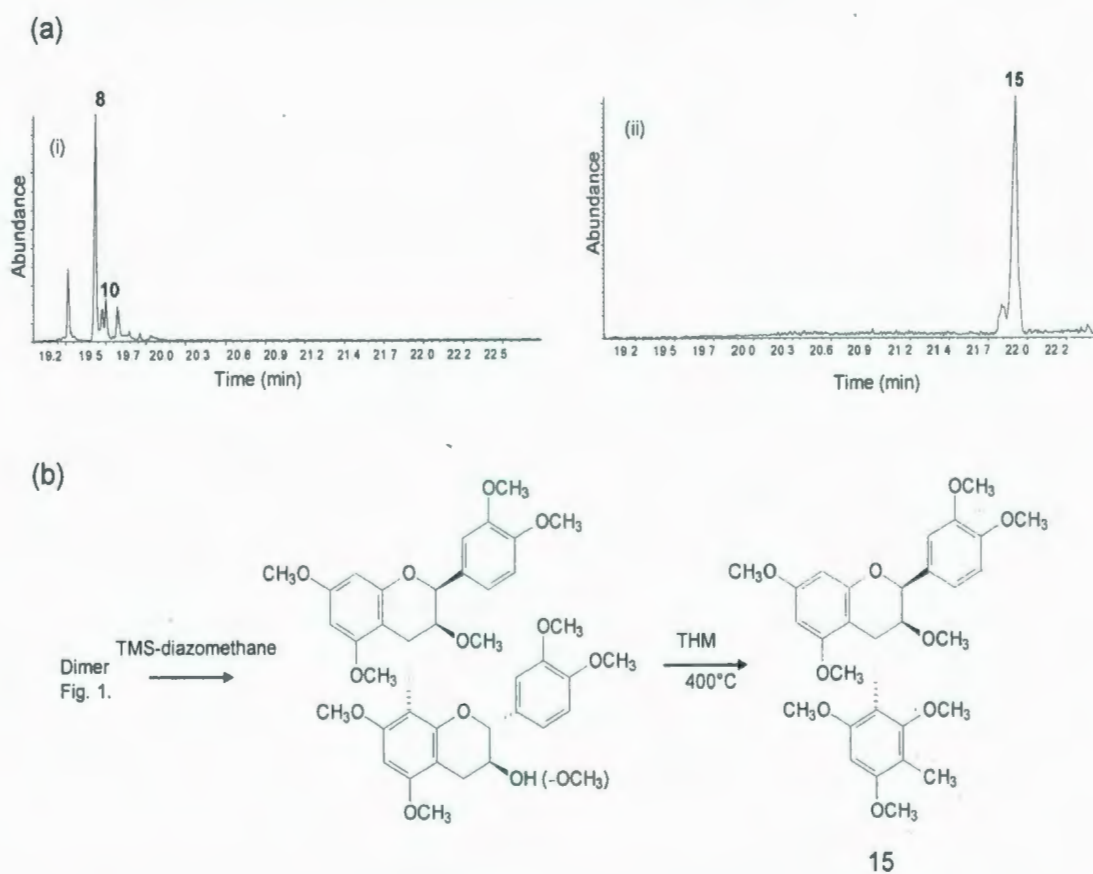


Fig. 3.10 (a) Partial total ion GC/MS chromatograms of dimer standard obtained by (i) THM (in presence of TMSH) at 250°C and (ii) two-step methylation at 400°C, (b) mechanism for formation of dimer marker (compound 15) by two-step methylation.

The resulting chromatogram from the two-step methylation procedure is shown in Fig. 3.10a(ii)). It is important to note that only a late-eluting (375°C) compound (**15**) was observed. It was also found that a much higher THM temperature was required to observe this product as shown in Fig. 3.10(b). The idea behind this approach was to reduce the reactivity of functional groups, to thermally stabilize them and to minimize isomerization in a dimer molecule before THM treatment. It was noted that the use of TMAH produced similar results as TMSH.

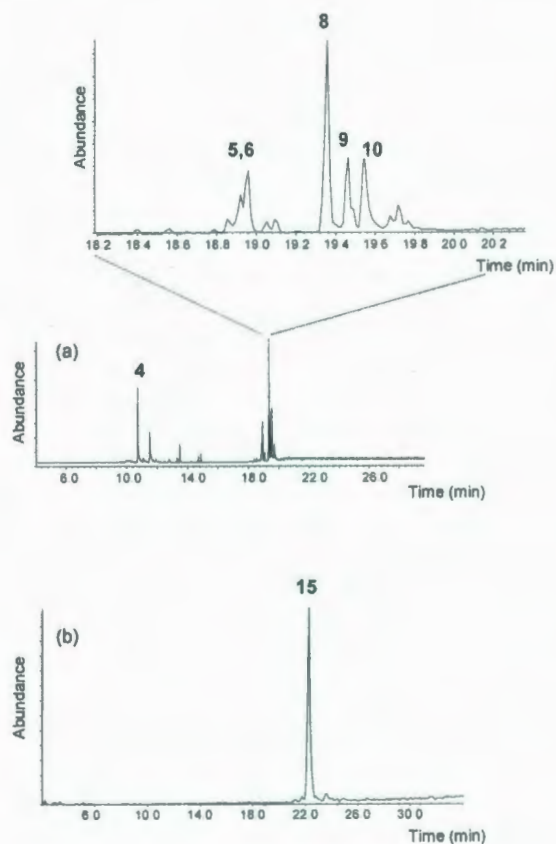


Fig. 3.11 Partial total ion chromatograms obtained from (a) THM (in presence of TMAH) at 250°C (b) two-step methylation at 400°C of kalmia condensed tannin.

The following describes the tentative identification of compound **15**. GC-MS in CI mode was not available due to the specialized high temperature column used in this study. The molecular weight of this compound was elucidated by DCI-MS analysis

where the $M+1$ ion was observed at 541. Furthermore, interpretation of the mass spectrum (EI mass spectrum Fig. 3.7(f)) is similar to the mass spectrum of fully methylated catechin (Fig. 3.7(a)) for m/z 150 to 194.

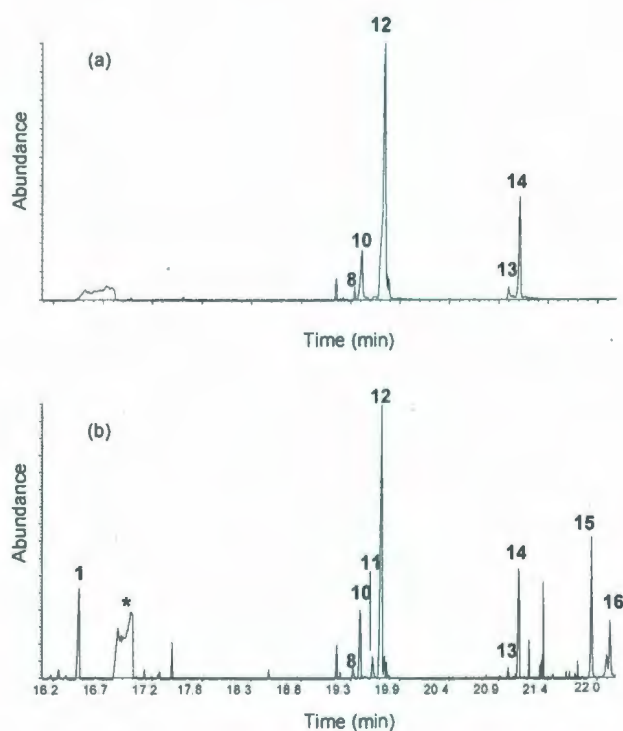


Fig. 3.12 Total ion chromatograms GC/MS obtained by (a) methylation using TMS-diazomethane and (b) two-step methylation of green tea extract at 400°C. * denotes contaminants.

The major fragment, m/z 194, is produced by Diels-Alder process discussed in the literature [37]. Interestingly, the proposed structure (15) includes a Diels-Alder fragment of the terminal catechin unit whereby the C-C interflavanoid bond is retained.

3.3.7 Real sample applications

To investigate applications of one step THM and the developed two-step methylation approach, condensed tannins from plant leaves including kalmia, blueberry and western hemlock, previously characterized by other workers [14], and green tea were analyzed.

3.3.7.1 Plant leaf tannins

Kalmia condensed tannin had a degree of polymerization of 2.3 elucidated from NMR studies in a previous study [14]. Terminal units were composed of 73% catechin and 27% epicatechin. The chromatogram resulting from THM (TMSH) of kalmia's tannin shown in Fig. 3.11(a), is similar to that of catechin. However, a noticeable difference in the peak intensity ratio of epicatechin marker (10) and catechin marker (8) indicates that another source of epicatechin other than from epimerization of catechin. The peak ratio is very closed to the 75:25 composition ratio of kalmia's condensed tannin terminal units. Thus, direct THM analysis of condensed tannins may allow for the measurement of terminal unit compositions.

Figure 3.11(b) shows the chromatogram resulting from two-step methylation of kalmia's tannin. The presence of a dimer (**15**) confirms the presence of catechin containing dimers as the DP was 2.3. The dimer was not observed unless high concentration of TMAH was used. Analysis of blueberry (DP=3.7) and western hemlock (DP=5.9) tannin showed decreasing amounts of the dimer marker.

3.3.7.2 Tea leaves

For tea leaves, a hot water extract was used to isolate condensed tannins and other polyphenols for ease of sample handling.

Previous studies [38] have found a large amount of catechin in green tea wherein catechin, epicatechin, gallic catechin and epigallocatechin were the major catechins. To the best of our knowledge, no dimer condensed tannins have been identified in tea leaves.

In order to determine the composition of phenolic monomers present in the green tea extract, TMS-diazomethane followed by GC-MS was undertaken. The difference in chromatographic results between this and two-step methylation analysis will assist in identifying tannin dimers and other non-monomeric constituents (i.e. gallic acid containing tannins).

The result of TMS-diazomethane GC-MS (Fig. 3.12(a)) showed the presence of high amounts of epigallocatechin (**12**) as reported by Nagle et al [39] and Graham [40] as the major catechin found in green tea. Also, similar to the findings of Chen et al [41], epicatechin (**10**) was the second most abundant flavan-3-ol in green tea. Also, in Fig.

3.12(a), two peaks (**13** and **14**) are associated with the group of flavonols; i.e. partially methylated kaempferol (**13**) and quercetin (**14**), which were identified in green tea by Wang and Helliwell [42].

The green tea extract was subjected to two-step methylation and the resulting chromatogram shown in Fig. 3.12(b), shows all the compounds observed by TMS-diazomethane analysis and many additional THM generated products. Our interest is the presence of two dimer condensed tannin markers (i.e. compound **15**, the marker for standard B1 dimer), and a new dimer marker (**16**), which has an identical mass spectrum to **15**. The second isomer, with a similar mass spectrum to B1, could be the product from B2 (epicatechin-(4 β -8)-epicatechin), B3 (catechin-(4 α -8)-catechin) or B4 (catechin-(4 α -8)-epicatechin) [43].

Finally, the presence of methylated gallic acid (**1**) and partially methylated gallocatechin (**11**) indicate that these moieties were initially bound in tannin structures since they were not observed as products with TMS-diazomethane treatment.

Direct Chemical Ionization (DCI)-MS experiments were performed on the products from off-line two-step methylation in order to verify the molecular weight of the products observed in GC (electron impact)/MS. The experimental set-up is similar to that of Helleur and Jackman [33]. Briefly, the pre-methylated green tea extract was deposited in a quartz pyrolysis tube, then, subjected to THM at 400°C. The resulting methylated products were analyzed by DCI-MS and the resulting total mass spectrum is shown in

Fig. 3.13. This figure represents the ion intensity of $M + 1$ of compounds 8, 14, 11, 7, 15 and 17 as a function of m/z , thus confirming their structures.

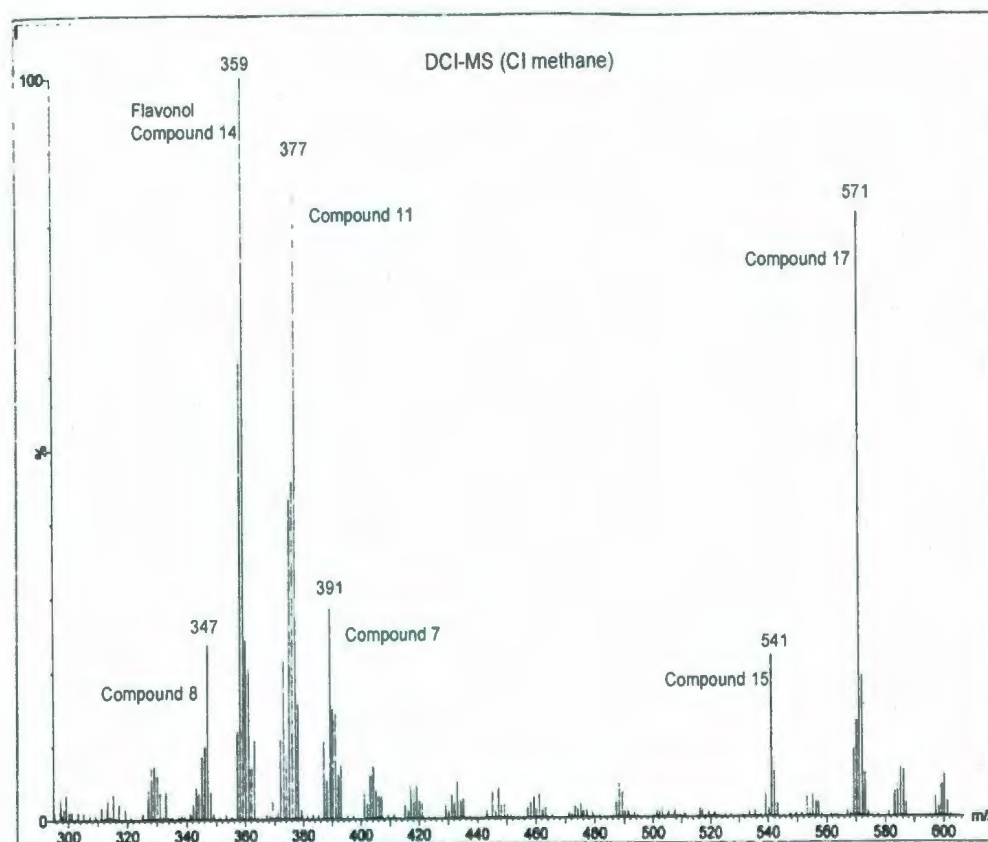


Fig. 3.13 Direct chemical ionization (methane) MS of products resulting from offline two-step methylation of tea extract (THM = TMSH at 400°C). See Fig. 3.6 for structures.

3.4 Conclusions

This study has advanced the use of THM-GC-MS for the analysis of catechins and condensed tannins by producing unique and intact 3-flavanol derivatives. Under careful temperature control, and, by using TMSH instead of TMAH, THM analysis of catechin, epicatechin and epigallocatechin all yielded their partially methylated forms. As well, THM of condensed tannins reveals the monomeric composition of its terminal units. Thus, in contrast to other THM/tannin studies, our analytical approach now allows for unambiguous identification for the presence of catechin compounds and their polymeric forms in complex samples.

For the purpose of identifying markers which retain the C-C interflavanoid linkage of condensed tannins, a two-step methylation technique was developed whereby the analyte was first pre-methylated using TMS-diazomethane, then subjected to THM under higher temperature. A high molecular weight marker ($m/z = 540$) can be observed indicating the presence of condensed tannin dimers but only if a high temperature capillary column is employed in GC analysis. The use of DCI-MS further revealed the presence of larger dimeric products when performing off-line THM of tea extracts. In contrast to catechin monomer analysis, sensitivity was improved if TMAH was employed in the second (THM) step.

Some limitations on THM-GC-MS of condensed tannins remain. The higher the average DP of tannins the less likely dimer markers will be observed by two-step methylation. As well, direct THM analysis only identified the terminal units of the tannin polymers. However, pre-methylation has been shown to stabilize the base-sensitive tannins and allows for detailed analysis of the complex stereochemistry of monomeric units and of interflavanoid linkage. Further studies of condensed tannins will be undertaken to exploit this.

3.5 Acknowledgments

We thank Dr. Caroline M. Preston for the purified condensed tannin samples which had an important role in our study. Funding from Natural Sciences and Engineering Research Council of Canada and Memorial University are acknowledged.

3.6 References

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Chapter 4: Use of an Injection Port for Thermochemolysis-Gas Chromatography/Mass Spectrometry: Rapid Profiling of Biomaterials

Farzad Shadkami and Robert Helleur*

Analytical Chemistry Group, Department of Chemistry, Memorial University of Newfoundland,

St. John's, Nfld, CANADA, A1B 3X7

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Abstract

A simple and direct approach was developed for thermochemolytic analysis of a wide range of biomolecules present in plant materials using an injection port of a gas chromatograph/mass spectrometer (GC/MS) and a novel solids injector consisting of a coiled stainless steel wire placed inside a modified needle syringe. Optimum thermochemolysis (or thermally assisted hydrolysis/methylation) was achieved by using a suitable methanolic solution of trimethylsulfonium hydroxide (TMSH) or tetramethylammonium hydroxide (TMAH) with an injection port temperature of 350°C. Intact methylated flavonoids, saccharides, phenolic and fatty acids, lignin dimers, and diterpene resin acids were identified. Samples include tea leaves, hemicelluloses, lignin isolates, and herbal medicines. Unexpected chromatographic results using TMAH reagent revealed the presence of intact methylated trisaccharides (658 amu) and structurally informative dimer lignin markers.

Keywords: Thermochemolysis; Trimethylsulfonium hydroxide (TMSH); Tetramethylammonium hydroxide (TMAH); Solids injector; Injection port; lignin markers.

4.1 Introduction

Thermochemolysis or Thermally Assisted Hydrolysis/Methylation (THM) is complementary to conventional direct pyrolysis and can provide more definitive structural information of the original composition of the sample. In thermochemolysis, heat is primarily used to drive the reaction between the methylating reagent and the acidic functional groups of the analytes while at the same time assisting in base-catalyzed cleavage of ester and ether bonds, and to a lesser extent, thermal fragmentation. Thermochemolysis has previously been used for analysis of biomaterials [1-4]. One of the common methylating reagents for thermochemolysis is tetramethylammonium hydroxide (TMAH), which originally was applied to analysis of fatty acids of triacylglycerols [5] whereby the fatty acids were determined as their methyl esters after cleavage of ester bonds of the triacylglycerides. Tetraethylammonium acetate (TEAAc) was also used for the derivatization of free fatty acids. Therefore, the combination of TMAH and TEAAc can be used to distinguish free fatty acids from covalently-bound fatty acids [6]. Similar to TMAH, trimethylsulfonium hydroxide (TMSH) has been used as an alternative methylating reagent. This reagent has been shown to be suitable for analytes which are alkaline- or heat-sensitive wherein it requires lower thermochemolysis temperatures and decreases the likelihood of base isomerization of polyunsaturated fatty acids [7]. Two further studies showed that TMSH was preferred to TMAH in the analysis of thermally-sensitive polyphenolics [4] and resin acids [1]. However, TMAH

has been shown to be useful for analysis of lignins when used at a much lower temperature than normal (310 °C v.s. 500°C) [8].

In most experiments, thermochemolysis has been implemented using pyrolysis devices such as the pyrolysis microfurnace [1,4], Curie-point [2], and heated filament [3] as the source of controlled and rapid heating. In a microfurnace pyrolyzer, the sample is dropped into the small hot quartz furnace by using a sample holder. In a Curie-point pyrolyzer the sample is placed onto a ferromagnetic wire and the wire inserted into the pyrolyzer. The wire is heated rapidly using a high frequency induction coil until the Curie-point temperature of the metal is reached. However, the selection of pyrolysis temperature is limited since it depends on the Curie-points of the available metals. In filament pyrolyzers, quartz tubes are used to hold the sample before being inserted into a Pt-coil filament. The Pt filament is rapidly heated by passing an electric current through the filament [9]. In the case of Curie-point and filament pyrolyzers, samples are unfortunately subjected to premature heating since they need to be first inserted into the preheated GC interface (i.e., 240-280°C). On the other hand, in the case of the microfurnace pyrolyzer, the sample is subjected only to the prescribed thermochemolysis temperature. The furnace can be set at a temperature lower than the GC interface since the interface is located after the microfurnace.

The limitations of all pyrolyzers are that they are external devices to a GC and need to be custom installed. In most situations, the GC needs to be dedicated. Finally, because the sample is introduced externally to the GC injection port, there is an inherent discrimination of high boiling point or high molecular weight products. The discrimination originates when products are transferred from the pyrolyzer to the GC injection port [1,10]. Efforts have been made to reduce this discrimination by the use of an in-column pyrolysis approach [11] whereby the sample was placed inside a disposable deactivated stainless steel tubing. One end of the tubing is connected to the GC injection port, the other to an analytical column. Since the sample holder is located inside the GC oven, this eliminates any cold spots which would cause condensation of products. Heating was initiated by passing an electric current pulse through the stainless steel tubing. Using this technique, it was shown that high molecular weight fatty acid methyl esters, which were discriminated by conventional pyrolysis, were observed by thermochemolysis in the presence of TMAH. This in-column pyrolysis was later redesigned using a septumless injector interface with an external pyrolysis tubing and a slot heater [12]. Beyer et al. [13] modified in-column pyrolysis by introduction of a valve before the analytical column; as a result, the solvent was removed without entering the analytical column.

A direct thermal desorption (DTD) interface was used for thermochemolysis of fatty acids [14, 15]. The DTD interface is an automated programmable temperature

vaporizer (PTV) interfaced with a GC, wherein the samples are put into a capped liner. Liners are heated in the desorption unit and a high gas flow causes the analyte to be transported to the GC injection port. A vacuum pump connected to a hole in the upper side of the liner was needed to dry the samples. In this setup, the liner is contaminated after each run and needs to be changed. To overcome this limitation, a small sample vial positioned inside the liner was used for TMSH thermochemolysis of fatty acids in human plasma and whole blood without any sample preparation [16]. A PTV interface was further modified by applying a column back-flush to avoid excessive amounts of reagent from entering the analytical column [17].

As discussed above, lower thermochemolysis temperatures can effectively be used for analysis of biomaterials, therefore suggesting the potential use of the GC injection port for thermochemolysis. Such an approach requires a method of solids introduction. The devices used include a solid Keele injector [18,19], a direct sample introduction (DSI) device [20], a syringless injector [21] and a solids injector commercially available from SGE, Australia. The SGE solid injector is a syringe-like device consisting of a thin metallic rod sample holder inside a syringe needle. Initial use of this device in thermochemolysis experiments suffered from severe blockage after just a few uses. The Keele injector is labor intensive since it requires samples to be sealed in glass ampules before introduction in the GC interface. The sample is released by breaking the ampule with a crushing rod. The DSI device uses a disposable glass vial

containing the sample which is then introduced into the GC injection port via a vial holder. The device requires one to purchase a modified GC injector. The commercially available syringless injector is a device that replaces the GC injection port liner to accommodate the sample vial to move vertically into and out of the injection port by controlling the split flow pressure. But the system requires a special adaptor and external controls of gas flows/pressures. Except for the SGE solid injector, other solids introduction devices require extensive modification of the GC injection port. They also require careful purging and handling in order to minimize contaminate and oxygen introduction.

Of noteworthy comment, thermochemolysis/derivatization using diazomethane [22] and phenyltrimethylammonium hydroxide [23] was successfully performed using a SPME fiber inside a GC injection port for the analysis of fatty acids present in solution. However, the authors did not mention how many times the same fiber can be used. It is likely that repeated use under the base conditions [23] required for thermochemolysis would damage its bonded phase. As well, this SPME/thermochemolysis approach is limited to analytes which are already in solution.

The GC injection port has been previously used as a heat provider device for derivatization [24-26] such as esterification of alkane sulfonate surfactants using tetrabutylammonium hydrogen sulfate [26] or the analysis of fatty acids in the presence

of TMAH [27,28]. In the present study, a novel thermochemolysis injection port method was tested on a wide range of plant compounds and compared to a previous study in which a microfurnace pyrolyzer was used to carry out thermochemolysis of polyphenolics [4]. It involves the simple design of a coiled solids injector along with a slightly modified GC injection port to function as a thermochemolysis device for direct analysis of natural compounds in plant material. The device consists of a coiled stainless steel wire (or solids injector) attached to a modified syringe.

4. 2 Experimental

4.2.1 Samples and reagents

All derivatizing reagents, i.e., methanolic solutions of TMAH (25% w/w), TMSH (0.25 M, ~2.5% w/w) and trimethylsilyl diazomethane in dimethylether (TMS-diazomethane, 2 M), and, standard samples of catechin, hypericin (98%) and arabinogalactan (98%) (hemicellulose from larchwood) were purchased from Sigma (St. Louis, MO, USA). The green tea leaves were President's Choice brand (Loblaws). For the preparation of green tea extract, the content of one tea bag was placed into a vial containing 10 ml of water, capped and heated for 10 min at 80°C. A 20 µl aliquot of tea extract was dried and used for thermochemolysis experiments. St. John's Wort tablets (Jamieson brand; Shoppers Drugmart stores) were ground before use. Freshly dried pine

needles (*Pinus strobes*) were ground to a fine powder and methanol/acetone (50/50) extracted. The cacao powder was a grocery store product and used as is. A kraft lignin sample was a gift from Dr. Sithole (Paper and Pulp Research Institute of Canada, Montreal).

4.2.2 Direct thermochemolysis solids injector

A direct thermochemolysis solids injector consisted of a stainless steel wire (0.25 mm id) which was coiled (as the solids injector) and attached to a modified Hamilton gastight syringe (1710RN, 100 μ L). The stainless steel wire moved freely inside a protective needle which was soldered to a luer attachment. Figure 4.1(a) shows the dimensions of the solids injector device in the retracted and injection positions. The septum had pre-drilled holes and was replaced after 20 injections due to high septum bleed.

A HP5890 GC injection port was modified by drilling a hole (1.55 mm id) in a septum retainer nut and an insert weldment (Figure 4.1(b)). A splitless liner (2 mm) was used since there are no solvents involved which resulted in rapid analyte transfer.

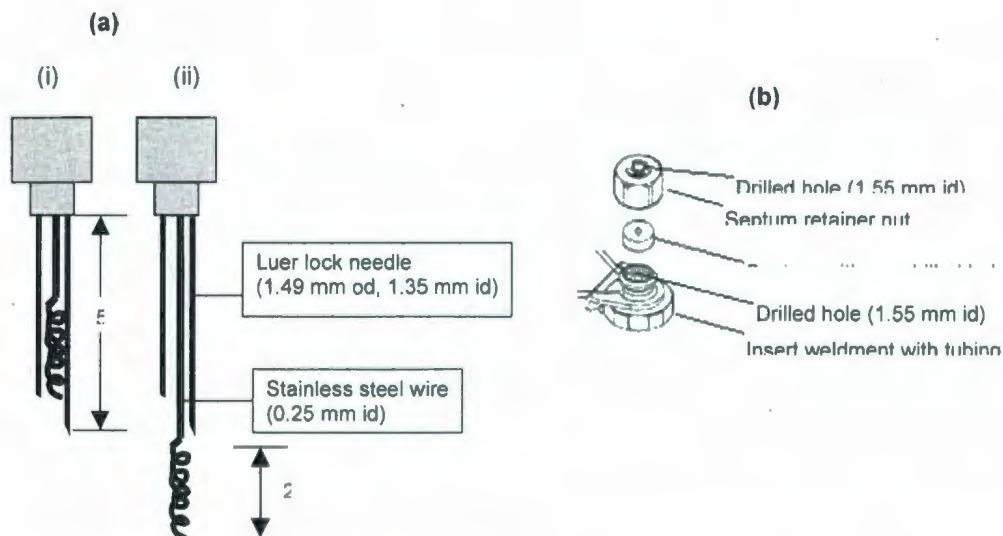


Fig. 4.1 (a) Scheme of a direct thermochemolysis solids injector. (i) retracted position (ii) injection position, (b) scheme of a modified GC injection port.

4.2.3 Thermochemolysis-gas chromatography-mass spectrometry

From previous work [4], optimum thermochemolysis procedures involved adding 500 μg of sample/standard to 2 ml amber vial and 30 μl of TMSH or TMAH was added. After vortex mixing, 5 μl of the solution/suspension was placed evenly over the surface of the coiled wire of the solids injector device and the methanol removed under a gentle flow of nitrogen. The device in the retracted position was introduced through the septum into the GC injection port (350°C). The coil was pushed down from the needle (Figure 4.1 (a)) and left in the hot injection region for 1 minute in a split flow mode. The column pressure was stabilized at 5 psi and the split flow was 10 ml min^{-1} . The HP5890 GC was equipped with an HP5971A mass spectrometer. A ZB-5HT Inferno capillary column (15

m, 0.32 mm id and 0.10 μm film thickness) was used. The GC injector was set at 350°C and the MS interface set at 300°C. Initial oven temperature was 100°C for 5 min then 20 °C min⁻¹ to 375°C, held for 5 min. The MS was operated in electron impact mode (70 ev) at 180°C source temperature. The MS scan range was set from 60 to 650 amu. Identification of the methylated products was assisted by the use of a NIST mass spectral library and from mass spectral data found in the literature (as indicated in Table 4.1).

4.2.4 Methylation using TMS-diazomethane

Previous work [4] used the methylating reagent TMS-diazomethane as a means of determining if the methylated thermochemolysis products were just originating from small molecules containing free phenolic or carboxylic acid groups or real thermochemolysis products requiring thermal bond fragmentation and/or base hydrolysis of ester or ether bonds. In the procedure, a 500 μg sample was treated with 30 μl of methanol followed by the addition of 30 μl of TMS-diazomethane. The sample was sonicated for 30 min. A 1 μl aliquot of the final solution was injected into the GC injection port (350 °C). This reagent was extensively used in the characterization of kraft lignin (see section 4.3.5).

4.3 Results and Discussion

A series of biomaterial samples were analyzed as a basis for studying the applicability of a direct thermochemolysis solids injector using an injection port of a gas chromatograph/mass spectrometer. Samples include cacao powder, herbal medicine St. John's Wort, green tea, a hardwood larch hemicellulose, dried pine needles, and lignin isolated from a kraft wood pulping process. As will be seen below, a wide range of intact methylated products were successfully observed including fatty acids, different flavonoids, sugars, diterpene resin acids, and lignin/lignan derivatives, some of which are illustrated in Figure 4.2. By using the direct thermochemolysis solids injector described above, suitable sensitivity and high analyte mass detection was achieved due to the proximity of thermochemolysis to the capillary column, the lack of solvent, the use of a 2 mm splitless liner, and the low split flow (10 mL min^{-1}). Sample preparation and injection took little time.

A limited series of replicate analyses ($n=3$) on two samples, catechin and the lignin sample, showed reproducibility in peak area of between 10-15%. Care must be taken to evenly apply the sample over the length of the solid injector coil.

The thermochemolysis chromatograms shown below are the result of experimental optimization where each type of sample was treated by TMSH and TMAH. The best

resulting chromatogram is shown in the figure and the reagent used indicated. The optimum thermochemolysis temperature for the largest TIC signal for all samples was found to be 350°C.

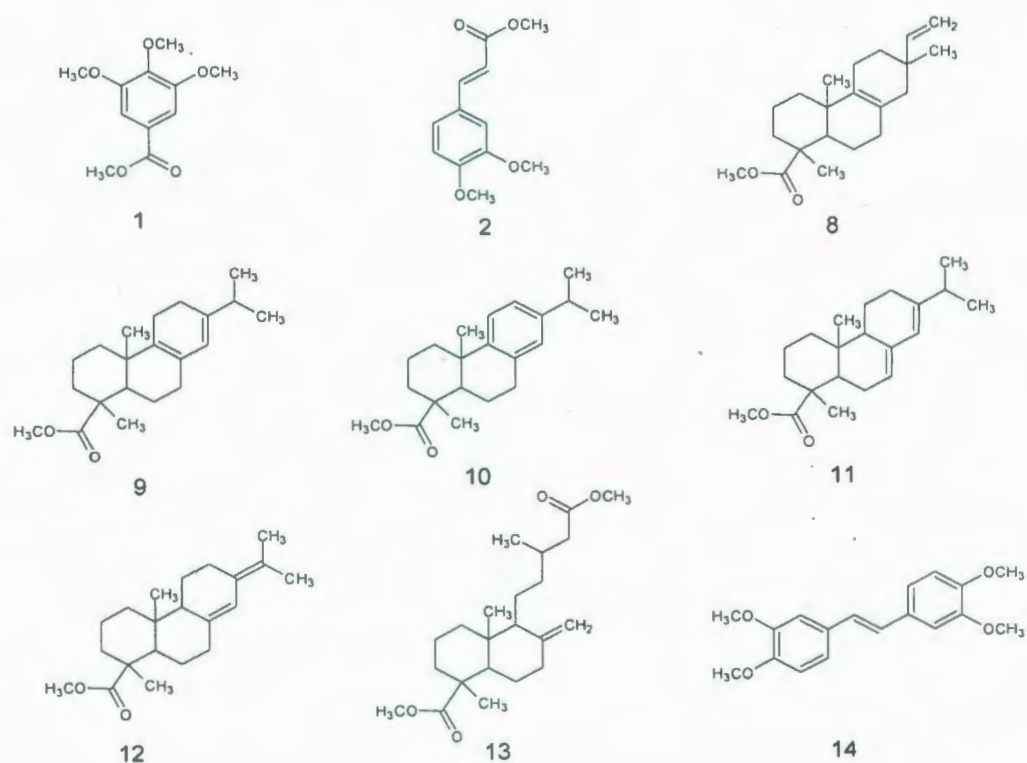


Fig. 4.2. Structures of thermochemolysis products identified by direct thermochemolysis-GC/MS as listed in Table 4.1.

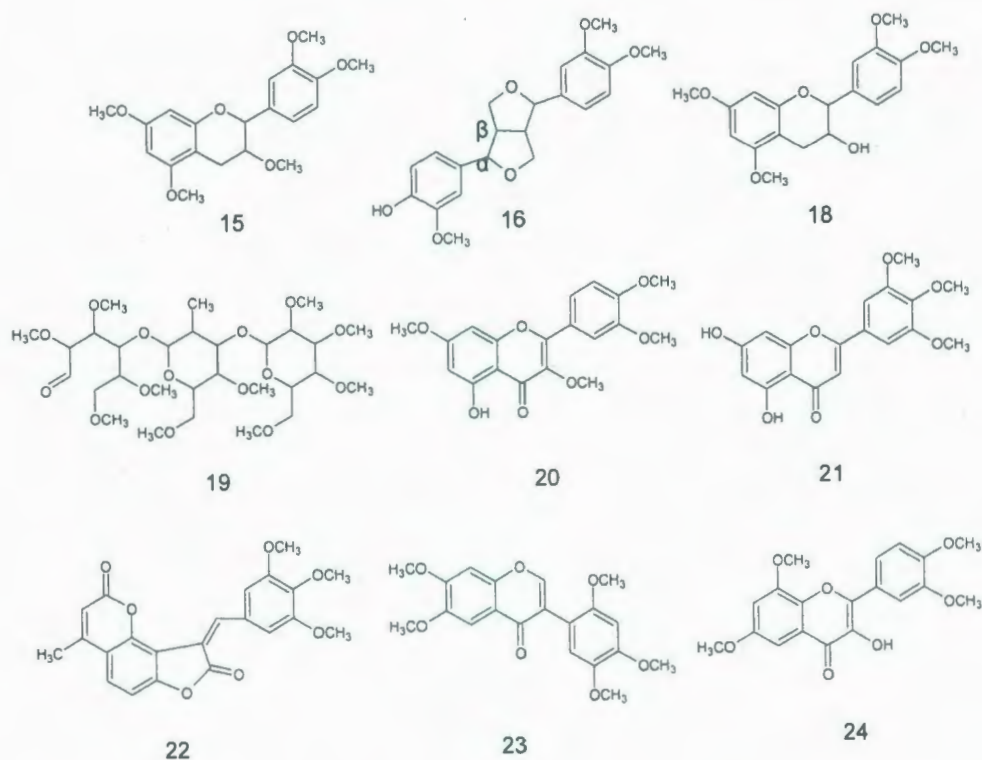


Fig. 4.2. Continued.

4.3.1 Catechin and green tea

Potential health benefits of green tea are mainly due to the antioxidant properties of its flavonoids [29]. Thermochemolysis of standard catechin, the main flavan-3-ol in green tea, was performed using the direct thermochemolysis solids injector in the presence of TMSH. The resulting chromatogram (Fig. 4.3(a)) is similar to the one from a previous study using a microfurnace pyrolyzer with similar conditions (350°C furnace

temperature) [4]. In this chromatogram, the fully methylated catechin (**15**) and partially methylated catechin (**18**) were observed. In the previous study using a microfurnace pyrolyzer, there was an additional chromatographic peak related to partially methylated epicatechin. This is believed to be a secondary product as the result of base-catalyzed isomerization. Under the present thermochemolysis conditions, this does not seem to occur.

The thermochemolysis of green tea in presence of TMAH (Fig. 4.3(b)) was successful and resulted in the identification of four different groups of compounds including phenolics and tannins, a flavanol, a flavonol and an isoflavone. Interestingly, no thermochemolysis products were produced when TMSH was used. Also, in our previous study using the microfurnace [4], no thermochemolysis products were observed (using TMAH) from the same green tea. Methylated gallic acid (**1**) was observed, most likely as a thermochemolysis product of epigallocatechin gallate, by cleavage of an ester bond [30]. Methylated catechin (**18**) is a thermochemolysis product of condensed tannins. Catechins are commonly found in green teas [31]. Compound **20** is associated with the group of flavonols, i.e. partially methylated quercetin, which was identified in green tea by Wang and Helliwell [32]. An isoflavone (**23**) is also observed in tea but there is no literature yet confirming this compound in tea. The last major product has an unknown structure. Its mass spectrum (**17**) is given in Figure 5(d).

Peak # ^d	M W	RT min.	Product ^e	Identification
1	226	3.01	3,4,5-Trimethoxybenzoic acid, methyl ester	a
2	222	6.02	2-Propenoic acid, 3-(3,4-dimethoxyphenyl)-, methyl ester	a
3	270	6.51	Hexadecanoic acid, methyl ester	a
4	292	7.62	9,12,15-Octadecatrenoic acid, methyl ester	a
5	294	7.63	6,9-Octadecadienoic acid, methyl ester	a
6	296	7.75	9-Octadecenoic acid, methyl ester	a
7	298	7.93	Octadecanoic acid, methyl ester	a
8	316	8.67	1-Phenanthrenecarboxylic acid, 7-ethenyl 1,2,3,4,4 α ,5,6,7,8,9,10,10 α ,dodecahydro-1,4 α ,7-trimethyl-methyl ester	a
9	316	8.75	Palustric acid, methyl ester	a
10	314	8.85	1-Phenanthrenecarboxylic acid, 1,2,3,4,4 α ,9,10,10 α ,octahydro-1,4 α -dimethyl-7-(1-methylethyl)-, methyl ester	a
11	316	9.04	Abietic acid, methyl ester	a
12	316	9.26	1-Phenanthrenecarboxylic acid, 1,2,3,4,4 α ,4 β ,5,6,7,9,10,10 α ,dodecahydro-1,4 α -dimethyl-7-(1-methylethylidene)-, methyl ester	a
13	364	9.68	1-Naphthalenepentanoic acid, decahydro-5-(methoxycarbonyl)5,8 α -trimethyl-2-methylene-, methyl ester	a
14	300	10.45	3,3',4,4'-Tetramethoxystilbene	a
15	360	10.82	Flavan, 3,3',4',5,7-pentamethoxy, trans/cis (fully methylated form of catechin)	b
16	372	11.18	Sylvatesmin	a
17	?	11.20	Unknown (spectra shown in Fig. 5.)	
18	346	11.47	3-Flavanol, 3',4',5,7-tetramethoxy, trans (partially methylated form of catechin)	a
19	658	11.84	2,3,5,6-Tetra-O-methyl-4-O-[2,4,6-tri-O-methyl-3-O-(2,3,4,6-tetra-O-methylhexopyranosyl)hexopyranosyl]hexose	a
20	358	11.99	Flavone, 5-hydroxy-3,3',4',7-tetramethoxy (quercetin 3,7,3',4', tetramethyl ether)	a
21	344	12.09	Flavone, 5,7-dihydroxy-3',4',5'-trimethoxy	a
22	394	12.26	9H-Furo[2,3-H]chromene-2,8-dione, 4-methyl-9-(3,4,5-trimethoxybenzylidene)	c
23	372	12.31	Isoflavone, 2',4',5',6,7-pentamethoxy	a
24	358	12.69	Flavone, 3-hydroxy-3',4',5,7-tetramethoxy	a

Table. 4.1. Chemical assignment of major chromatographic peaks

^a NIST library, ^b from ref. 4, ^c tentative, ^d as shown in chromatograms.

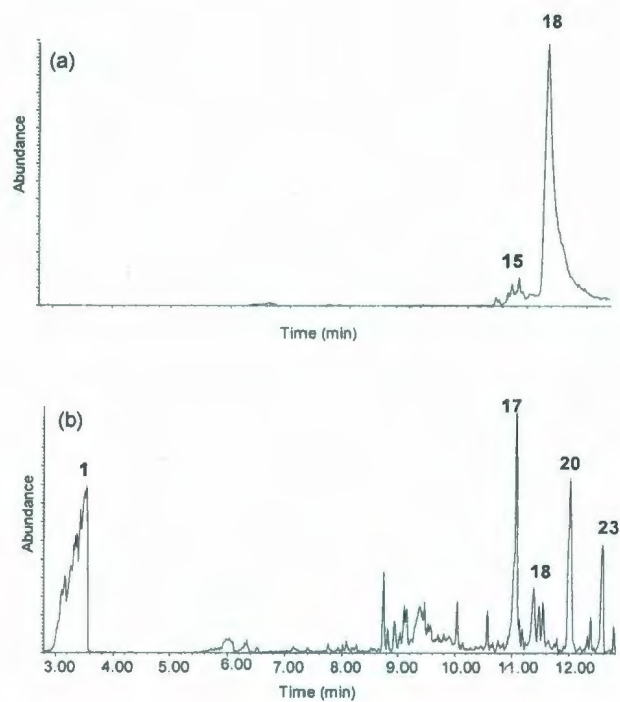


Fig. 4.3. Partial total ion chromatograms obtained from thermochemolysis of (a) catechin and (b) green tea at 350°C.

4.3.2 Cacao

Cacao powder contains high levels of flavan-3-ols and fatty acids [33,34]. Thermochemolysis of cacao using TMSH revealed three fatty acids; hexadecanoic acid (palmitic acid) (3), 9-octadecenoic acid (oleic acid) (6) and octadecanoic acid (stearic acid) (7) as shown in Figure 4.4(a). The presence of these fatty acid methyl esters only, indicates that no unwanted base-catalyzed isomerization occurred. These fatty acids were reported as the major lipid components in ripe cacao beans [34]. A small amount of fully methylated catechin/epicatechin (15), was also observed in the thermochemolytic analysis thus confirming the presence of flavan-3-ols in cacao.

4.3.3 St. John's Wort

St. John's Wort (*Hypericum perforatum*) is a widely used herbal (flower) medicinal plant. The thermochemolysis products in the presence of TMSH as shown in Figure 4.4(b) can be categorized into small phenolic compounds, fatty acids and flavonoids. From the literature, it has been found that caffeic and chlorogenic acids, fatty acids and flavonoids are present in this plant preparation [35]. In the present chemical analysis, the source of 2-propenoic acid, 3-(3,4-dimethoxyphenyl)-, methyl ester (2), a methylated cinnamic acid, can be from caffeic acid or it might be the thermochemolysis product of chlorogenic acid by the cleavage of an ether bond. Major fatty acids include hexadecanoic acid (palmitic acid) (3) and octadecanoic acid (stearic acid) (7) which have been reported in St. John's Wort [36]. Product 20, a methylated quercetin derivative,

may originate directly from methylation of quercetin, or, it can be from the thermochemolysis of quercetin-containing rutin through the cleavage of the sugar linkage [37]. Compounds **21**, a myricetin derivative, and **24**, a flavone, have previously been found in this herbal plant [38]. Compound **23** has been identified as a methylated isoflavone but no previous study has discovered its presence in this plant.

An additional experiment was undertaken to analyze the hypericin standard. It is the major polyphenolic and active medical ingredient found in St. John's Wort [39]. Thermochemolysis with either TMSH or TMAH resulted in no products which indicates that none of the above phenolic compounds in Fig 4.4(b) were fragmentations of hypericin. Thermochemolytic analysis of St. John's Wort can afford a useful way for authentication and quality control of this and other herbal formulations based on the wide variety of thermochemolysis markers.

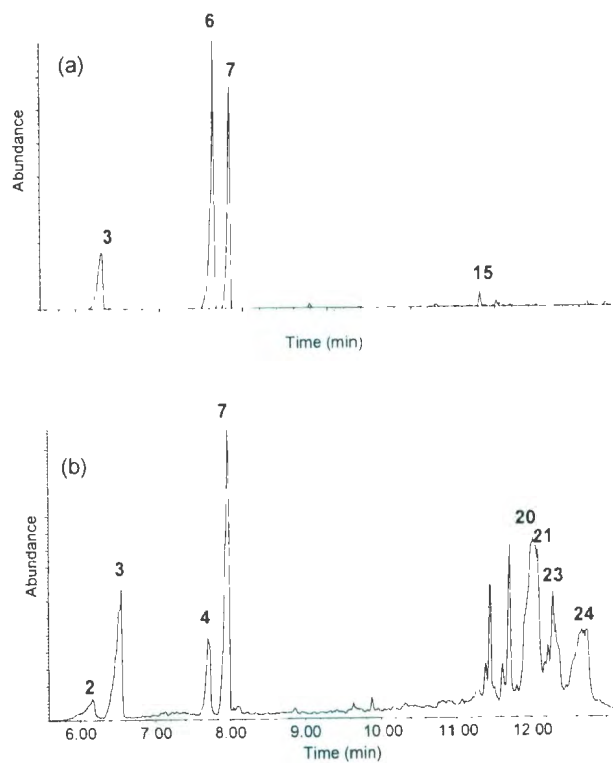


Fig. 4.4. Partial total ion chromatogram obtained from thermochemolysis (in presence of TMSH) of (a) cacao and (b) St. John's Wort at 350°C.

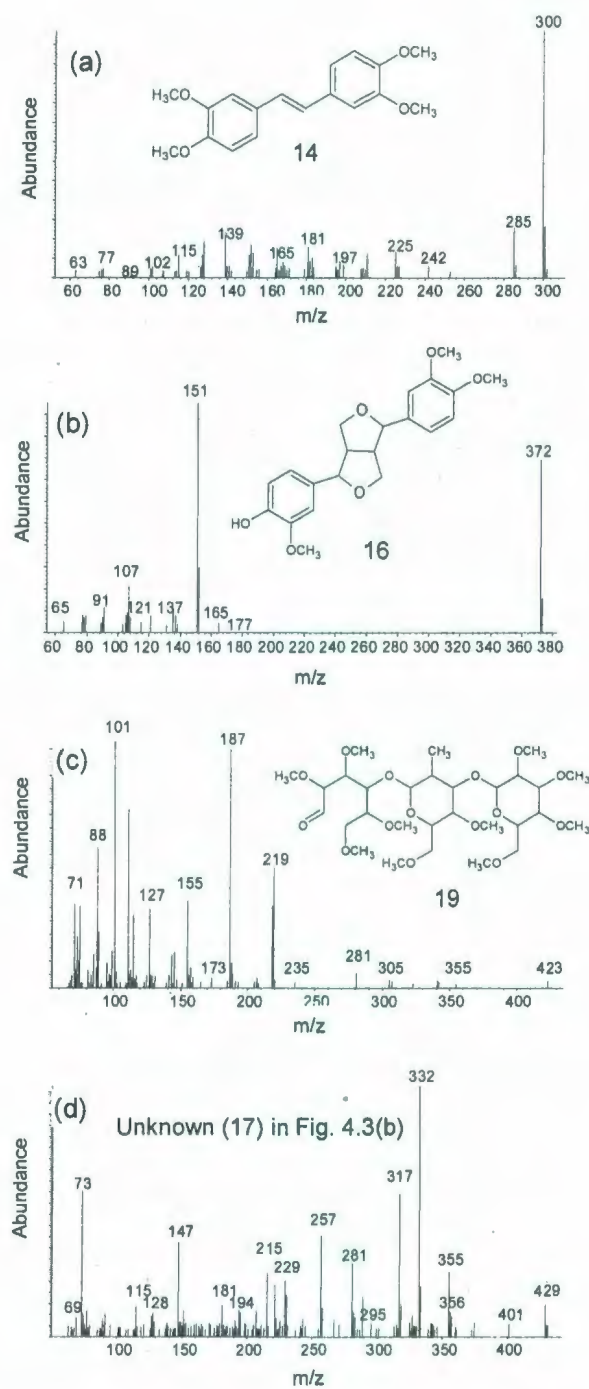


Fig. 4.5. EI mass spectra of methylated products (as listed in Table 1); 14 (a), 16 (b), 19 (c) and unidentified product 17 (d) in green tea (Fig. 4.3(b)).

4.3.4 Hardwood larch hemicellulose

Hemicelluloses are polysaccharides derived from different sugar units and constitute the major biomass of most plants. An example is arabinogalactan, a polysaccharide made of galactose and arabinose sugar units and present in the larch tree. The significant antimicrobial activity of arabinogalactan makes the screening of this compound important [40].

One of the first thermochemolytic studies of carbohydrates was undertaken in the presence of TMAH at 700°C [41]. Analysis of mono and oligosaccharides yielded the saccharides own unique permethylated deoxy aldonic acids as well as producing benzene derivatives as products, i.e. 1,2,4-trimethoxybenzene, 2,4-dimethoxyphenol. For polysaccharides such as starch and cellulose, 1,2,4-trimethoxybenzene was the only major thermochemolysis product.

In the present study the analysis of arabinogalactan in the presence of TMAH (350°C) resulted in a methylated oligosaccharide product (19) as observed in Figure 4.6(a). This is truly a high molecular weight marker (MW = 658 amu) containing two hexopyranosyl rings attached to a hexose structure. This is also the first time an oligosaccharide product has been observed by thermochemolysis. Its mass spectrum can be found in Figure 4.5(c). The intact methylated oligosaccharide may not have been observed by other

thermochemolysis approaches, likely due to the high molecular weight discrimination using traditional pyrolyzers. Interestingly, this study showed no low molecular weight carbohydrate markers such as those produced in a previous study [41]. Because of a lower thermochemolysis temperature (350°C vs 700°C), low molecular weight compounds resulting from excessive decomposition were not observed. In addition to product **19**, there were three other oligosaccharide peaks (marked by * in Figure 4.6) based on the presence of the large $m/z = 101$ (methylated hexose fragment) in their mass spectrum.

4.3.5 Kraft lignin

Kraft lignin, a highly cross-linked polyphenolic, is the product of the kraft pulping process whereby lignin from wood is extracted by alkali treatment [42]. Figure 4.6(b) represents the chromatogram resulting from thermochemolysis of kraft lignin in the presence of TMSH. It appears that this analysis approach is also suitable for the monitoring of the common lipophilic extractives in softwood lignin. In softwoods, the lipophilic extractives include resin acids and triacylglycerols [43, 44]. Compound **5**, a fatty acid methyl ester, was also identified in another study, which performed thermochemolysis of triacylglycerides in leaf litter [45]. The resin acid methyl esters of pimaric acid (**8**), dehydroabietic acid (**10**), abietic acid (**11**), and neoabietic acid (**12**) are all common in softwoods [46]. These resin acids must have been bonded through their

ester bonds in the lignin molecules before analysis. This was confirmed when an additional experiment was performed whereby simple methylation of the lignin sample with trimethylsilyl diazomethane (see section 4.2.4) did not result in any methylated resin acid products.

Thermochemolysis of kraft lignin produced a number of lignin phenolic markers. Compound **14**, methylated stilbene, was previously identified as a TMAH thermochemolysis product of wood extractives originating from lignin [47-48]. The mass spectrum of compound **14** is given in Figure 4.5(a). The molecular ion is m/z 300 while the fragment ions of m/z 285, 255 and 225 are formed from the elimination of a methyl group, plus a methoxy group, plus a second methoxy group, respectively.

The lignin dimer (**16**) with its mass spectrum shown in Figure 4.5(b) is called by the trivial name, sylvatesmin [48]. It contains two fused tetrahydrofuran rings bonded to two terminal phenol rings. The original structure of this thermochemolysis product has been suggested to exist as guaiacyl β - β subunits in the lignin polymeric structures [49]. Before thermochemolysis, one of the phenolic groups from one or both of the aromatic rings are originally attached to the lignin macrostructure then cleaved during thermochemolysis process.

Compound (22) is another major phenolic marker produced from thermochemolysis of kraft lignin. This compound is formed from two different lactone type structures (i.e. γ and δ). The γ type was previously found by other workers as a lignin oxidation product from straw [50] and as lignan in Finland spruce knots [46]. It seems that the aromatic group on the right side (see Figure 4.2) is part of the oxidation product of a methylated sinapyl alcohol, one of the constituent lignin precursors [51].

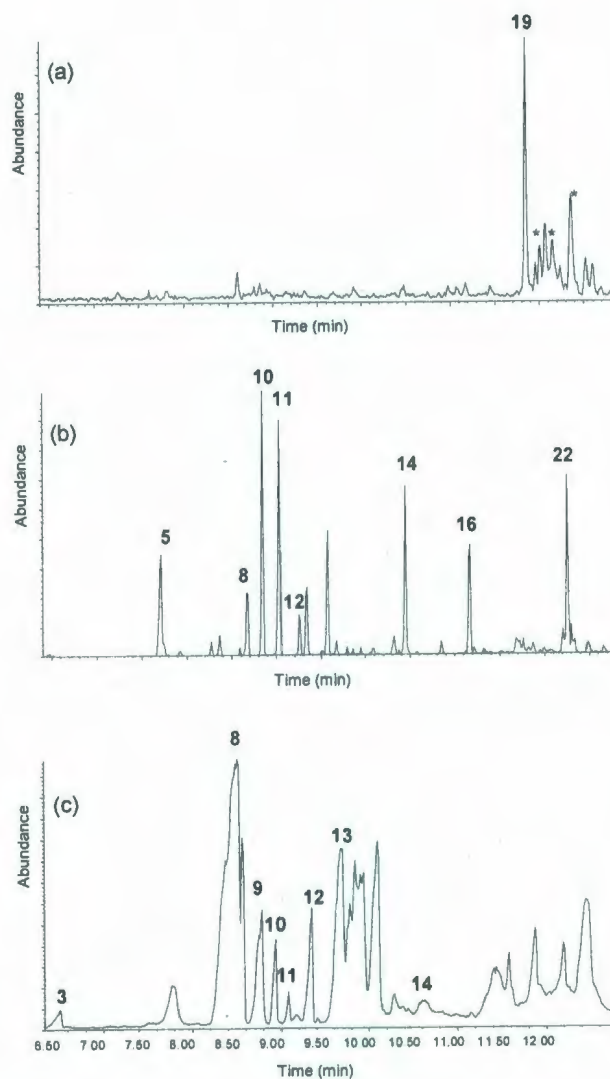


Fig. 4.6. Partial total ion chromatograms obtained from thermochemolysis (a) in presence of TMAH of a hardwood larch hemicellulose. * related to sugars, (b) in presence of TMSH of kraft lignin and (c) in presence of TMSH of pine needles at 350°C.

4.3.6 Pine needles

Forestry studies have long been interested in tree needle analysis in terms of forest health. In one study, the concentrations of resin acids were measured in pine needles as a proxy of nitrogen availability [52] and in another, the analysis of lipid and resin acids in pine needles of *Pinus strobes* [53]. Thermochemolysis in the presence of TMSH of needles (*Pinus strobes*) yielded mainly diterpene resin acid methyl esters (Fig. 4.6(c)) which have been found in previous studies of this pine tree [52, 53]. These include the methyl esters of pimaric acid (8), palustric acid (9), dehydroabietic acid (10), abietic (11) and neoabietic acid (12). A unique product containing two carboxylic acid methyl ester groups with a naphthalenepentanoic acid structure (13) and a common lignin marker, product 14, were also identified.

4.4 Conclusions

It has been demonstrated that, by using a direct thermochemolysis solids injector and a GC injection port, favorable thermochemolysis products can be observed. Not only are the results similar to those using a traditional microfurnace pyrolyzer but it carries some clear advantages. The close proximity of the thermochemolysis zone in the injection port to the column allows for observation of higher molecular weight products and better sensitivities. As well, simple modification is required to turn any GC into a

thermochemolytic apparatus for carrying out analysis with minimum sample preparation. In terms of real samples, a wide range of compounds can be screened including phenolics, fatty acids, diterpene resin acids and flavonoids as their methylated derivatives. This is the first time that green tea has been successfully analyzed for a number of its components observed in its chromatogram. Flavan-3-ols have been positively identified in tea and cacao and the analysis of a herbal medicine revealed a number of unique fatty acids and flavonoids, useful as authentication markers. In addition, a large methylated oligosaccharide (658 amu) and a number of dimer lignin markers were observed in other plant samples. From the success of observing large lignin markers additional work will be carried out in thermochemolytic applications of profiling lignins from different sources.

4.5 Acknowledgments

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Chapter 5: Rapid Screening of Hardwood and Softwood Lignins by Low Temperature Thermochemolysis Using a GC Injection Port

Farzad Shadkami^a, Bruce B. Sithole^b, Robert Helleur^{a*}

^aBiomass Chemistry Group, Department of Chemistry, Memorial University of Newfoundland, St.

John's, NL, CANADA, A1B 3X7

^bPaper and Pulp Research Institute of Canada, 570 boul, Saint-Jean, Pointe-Claire, QC,

CANADA, H9R 3J9

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Abstract

A low temperature thermochemolysis method was developed to rapidly differentiate softwood and hardwood lignins. The technique uses heat at 350°C in the presence of trimethylsulfonium hydroxide (TMSH) whereby the sample is introduced via a fabricated solids injector into the injection port of a gas chromatograph-mass spectrometer (GC-MS). No major modification or external devices are required for instrument conversion and the switchover to normal use is done in minutes. Three different softwood and two hardwood lignins were examined. All the resultant thermochemolysis results revealed either yangambin, a hardwood dimeric marker containing syringyl groups, or sylvatesmin, a softwood dimeric marker containing guaiacyl groups. The injection port results were confirmed by thermochemolysis using a traditional microfurnace pyrolyzer. This study also revealed the presence of 3,3',4,4'-tetramethoxystilbene, a marker for both types of lignin. It appears that the thermochemolysis-TMSH technique in a 350°C (max) injection port produces larger methylated units of the lignin substructures than those obtained by thermochemolysis in the presence of the more common methylating agent of tetramethylammonium hydroxide (TMAH) which is often used at much higher temperatures. In addition to lignin markers other compounds such as resin acid, methyl esters were liberated but only as the result of thermochemolysis. These materials were not observed by solvent extraction-methylation

which indicates that these resin acid units may be covalently bound to the softwood lignin macrostructure.

Keywords: thermochemolysis; trimethylsulfonium hydroxide; injection port; sylvatesmin, yangambin, lignin marker, lignin; solids injector; softwood; hardwood; pyrolysis.

5.1 Introduction

Lignin is the most abundant phenolic compound and it is the main constituent of cell walls in plants after cellulose. The main monomeric components of the polymeric structure of lignin were proposed as phenylpropanyl units (C_6-C_3) (Adler, 1977; Banoub and Delmas, 2003). This includes *p*-coumaryl alcohol (or *p*-hydroxy-cinnamyl alcohol), coniferyl alcohol (or guaiacyl alcohol), and sinapyl alcohol (or syringyl alcohol) and their aldehyde derivatives. It has been shown that these structures vary between plant species (Monties, 1989) trees (Musha and Goring, 1975) and even within the tree itself (Ona et al., 1997). Different substructures for lignins have been proposed such as those in Figure 5.1 including (a) β -aryl subunit (Kuroda and Nakagawa-izumi, 2005) (b) β -ether subunit (Clifford et al., 1995) (c) β - β subunit (Nakagawa-izumi et al., 2004) and (d) β -5 subunit (Kuroda and Nakagawa-izumi, 2006a).

A variety of techniques has been used to ascertain the structure of lignin polymers. Hardwood, softwood, and grass lignins were cleaved by pyrolysis and the products compared (Saiz-Jimenez and De Leeuw, 1986). Softwood lignin yielded coniferyl derivatives, hardwood lignin coniferyl, and sinapyl derivatives and grass lignin, *p*-vinylphenol as major biomarker pyrolysis products. A disadvantage of conventional direct pyrolysis is that the pyrolysis products are not derivatized;

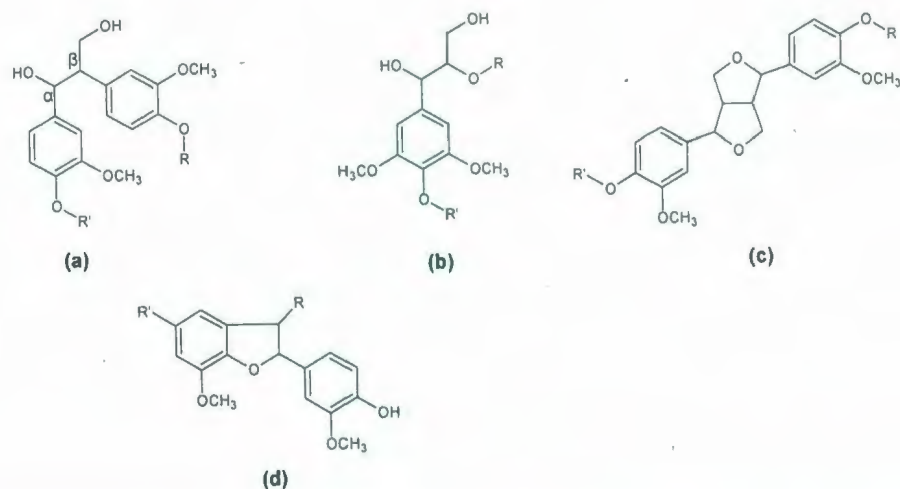


Fig. 5.1 Examples of lignin substructures (a) β -aryl subunit, (b) β -ether subunit (c) β - β subunit and (d) β -5 subunit

consequently, the fragments which contain polar functional groups exhibit poor chromatographic behavior. Sonoda et al. (2001) improved this by acetylation of functional groups in the lignin prior to pyrolysis. An added advantage of this approach is

the prevention of secondary formation of cinnamaldehyde from the corresponding alcohol which occurs during pyrolysis. The structure elucidation of lignins can also be done by degradation to low molecular weight compounds via cleavage of ether bonds (mainly β -O-4 or C₈-O-4') by thioacidolysis (Rolando et al., 1992). Drawbacks of this technique are incomplete cleavage and side chain degradation (Lu and Ralph, 1997). Other studies of lignins are based on determination of dimers, trimers and higher linkages in the polymers using mass spectrometry. Recently, Banoub et al. (2007) studied wheat straw lignin by atmospheric pressure photoionization quadrupole time-of-flight mass spectrometry (APPI-QqTOF-MS). This thorough study gave evidence that grass lignin is composed of repeating phenylcoumaran units which are formed from two di-coniferyl units linked by the C₈-C₅ covalent bond and the ether C₇-O-4' linkage forming a furan-like ring attached to an aromatic coumaran ring. Evstigneyev et al. (2004) characterized the structure of dimeric lignin model compounds by polarography. These structures include the presence of a furan-like ring attached to an aromatic ring and diphenyl dimers attached through a carbon-carbon bond or an ethene bond which forms a stilbene-like molecule.

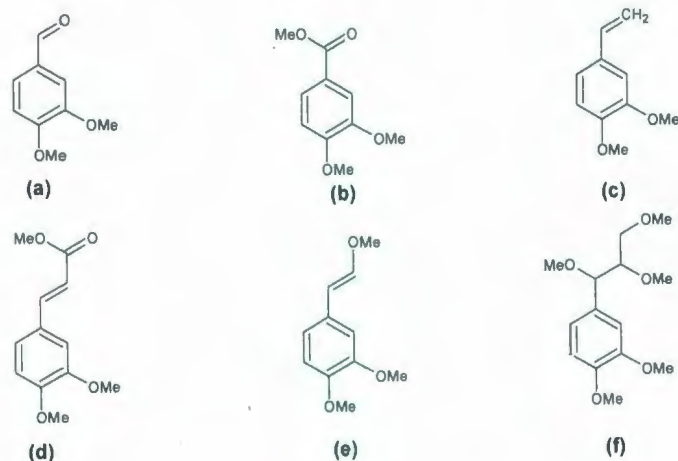


Fig. 5.2 Structures of common lignin thermochemolysis products (a) 3,4-dimethoxybenzaldehyde (b) 3,4-dimethoxybenzoic acid methyl ester (c) 3,4-dimethoxystyrene (d) 3,4-dimethoxycinnamic acid methyl ester (e) 1-(3,4-dimethoxyphenyl)-2-methoxyethene and (f) 1-(3,4-trimethoxyphenyl)-1,2,3-trimethoxypropane.

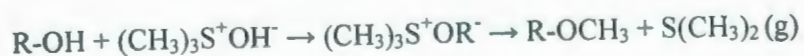
Another useful technique is thermochemolysis or thermally assisted hydrolysis and alkylation, usually methylation. In thermochemolysis, heat is primarily used to drive the reaction between the methylating reagent and the acidic functional groups of the analytes resulting in the formation of the methyl esters of the carboxylic acids and methyl ethers of the phenols. Moreover, at the same time, heat assists in base-catalyzed cleavage of ester and ether bonds, and to a lesser extent thermal fragmentation (Shadkami and Helleur, 2009). Thermochemolysis of lignins were studied using tetramethylammonium hydroxide (TMAH) (Clifford et al., 1995; McKinney et al., 1995; Klingberg et al., 2005).

McKinney et al. (1995) found 3,4-dimethoxybenzaldehyde, 3,4-dimethoxyacetophenone, 3,4-dimethoxybenzoic acid methyl ester, and 3,4-dimethoxystyrene as the main lignin thermochemolysis products (see Fig. 5.2). Clifford et al. (1995) also found methylated coniferyl derivatives resulting from thermochemolysis by the cleavage of β -O-4 bonds.

Kuroda and Nakagawa-izumi (2006b) performed thermochemolysis of softwood lignin in the presence of TMAH at much lower temperature of 315°C, resulting in two new markers, 1-(3,4-dimethoxyphenyl)-2-methoxyethene (Fig. 5.2) and 1-(3,4-dimethoxyphenyl)-1,2,3-trimethoxypropane, both derived from β -aryl ether subunits. In another study, guaiacyl-syringyl mixed polymer (a model compound) was subjected to thermochemolysis in the presence of TMAH (Kuroda and Nakagawa-izumi, 2005). The products included (a) 1-(3,4-dimethoxyphenyl)-1,2,3-trimethoxypropane and 1-(3,4,5-trimethoxyphenyl)-1,2,3-trimethoxypropane as β -aryl ether subunit-derived products (b) 2,3,3',4'-tetramethoxy-5-(3-methoxyprop-1-enyl)stilbene and 2,3,3',4',5'-pentamethoxy-5-(3-methoxyprop-1-enyl)stilbene as β -5 subunit-derived products, (c) pinoresinol dimethyl ether and syringaresinol dimethyl ether as β - β subunit-derived products and (d) coniferyl alcohol dimethyl ether and sinapyl alcohol dimethyl ether from 4-O-linked coniferyl and sinapyl alcohols. A study by Ishida et al. (2007) incorporated the use of tetrabutylammonium hydroxide (TBAH) as the alkylating reagent. The advantage of using this reagent over TMAH is that original methyl groups present in the lignin can be differentiated from the butyl groups introduced by the alkylation. For example, 3,5-

dimethoxy-4-butoxybenzoic acid butyl ester and 3,4,5-tributoxybenzoic acid butyl ester, both gallic acid derivatives, were able to be discriminated. The first compound was a lignin marker and the second a hydrolysable tannin marker. For the same reason ^{13}C -TMAH was used in thermochemolysis GC/MS of lignin samples (Filley, et al., 2006) where it was ascertained that the order of monohydroxyl (i.e. 3,5-dimethoxy, 4-hydroxyl in source) content of syringyl compounds in oak was leaves (70%) < root (75%) < bark (77%) < brown rot wood (86%) < wood (89%).

Trimethylsulfonium hydroxide (TMSH) has been used as a thermochemolytic reagent (Vosmann et al, 1997, Shadkani and Helleur, 2009). Since it requires lower thermochemolysis temperatures for methylation, its use decreases the likelihood of base isomerization of polyunsaturated fatty acids (Estevez and Helleur, 2005) and should minimize base catalyzed decomposition. It also was preferable to TMAH for the analysis of thermally-sensitive polyphenolics (Shadkani et al., 2009) and resin acids (Shadkani et al., 2007). The suggested mechanism for phenolic (or carboxylic acid) reaction with TMSH (Shadkani et al. 2009) is:



In cases where ester or ether bonds are present, the analyte first undergoes bond hydrolysis with subsequent formation of the sulfonium salt.

Another mechanism was suggested as follows (Sithole, 2000):



In the later mechanism trimethylsulfonium hydroxide undergoes bond breakage (C-S) under heat. Methylcarbonium is formed which reacts with a phenolic (or a carboxylic) group producing the methoxy group. In the other mechanism, it is suggested that the TMS salt occurs first after which methylation occurs at higher thermochemolysis temperatures.

The present study contributes to the discovery of lignin markers in hardwood and softwood lignins by direct TMSH thermochemolysis using a fabricated solids GC injector device (Shadkami and Helleur, 2009). The approach is rapid, inexpensive and useful in distinguishing between hardwood and softwood lignins.

5.2 Experimental

5.2.1 Samples and reagents

All reagents, i.e., methanolic solutions of TMAH (25% w/w), TMSH (0.25 M, ~ 2.5% w/w) and trimethylsilyl diazomethane in dimethylether (TMS-diazomethane, 2 M)

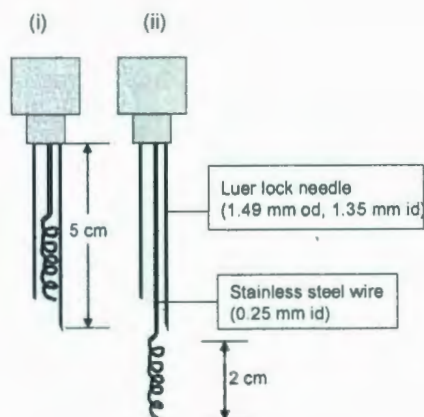


Fig. 5.3 Design of a direct thermochemolysis solids injector, (i) retracted position and (ii) injection position.

were purchased from Sigma (St. Louis, MO, USA). Lignin samples were purchased (Sigma) or obtained from researchers at pulp and paper institutions. More detailed information as to the source and chemical nature of the individual lignins are discussed in the Results and Discussion section.

5.2.2 Direct thermochemolysis solids injector

A direct thermochemolysis solids injector shown in Figure 5.3 consisted of a coiled stainless steel wire (0.25 mm id) (as the solids injector) that was attached to a

modified Hamilton gastight syringe (1710RN, 100 μ L). The stainless steel wire was protected inside a needle (with a luer attachment). More details of the device was reported in chapter 4.

5.2.3 Thermochemolysis-gas chromatography-mass spectrometry using a direct solids injector

In thermochemolysis procedures, 500 μ g of sample were initially placed in a 2 ml amber vial and 30 μ l of TMSH was added. After sonication of the solution/suspension for 2 min, 5 μ l of the mixture was placed all along the surface of the coiled stainless steel wire of the solids injector using a 10 μ l syringe, then purged to dryness under N_2 . The solids injector in a retracted position was introduced into the GC injection port (350°C); the needle was pushed down and remained in the injection port for 1 minute (chapter 4). The HP5890 GC-MS was equipped with an HP5971A mass spectrometer (EI mode). A ZB-5HT Inferno capillary column (15 m, 0.32 mm id and 0.10 μ m film thickness) was used. The GC injector was set at 350°C and the MS interface was set at 300°C. The column pressure was adjusted to 5 psi and split flow at 10 ml min⁻¹. Oven temperature programming: 100°C for 5 min then 20°C min⁻¹ to 375°C, held for 5 min. The mass spectra scan ranged from 60 to 650 amu. Identification of methylated products was found using the NIST MS library and from spectra in the literature.

5.2.4 Thermochemolysis-gas chromatography-mass spectrometry using a microfurnace pyrolyzer

In all thermochemolysis procedures, 500 μg of samples were placed in a 2 ml amber vial and 30 μl of TMSH or TMAH were added. After mixing, 5 μl of the solution were transferred to a sample cup and the solvent evaporated under N_2 . An auto-shot sampler (AS-1020E, Frontier Lab) was used to introduce the sample to a vertical double-shot micro-furnace pyrolyzer (PY-2020iD, Frontier Lab). A disposable sample cup (a small u-shape stainless steel sample cup) was loaded into the carousel which rotates after each analyte. The sample/reagent mixture was then subjected to heat automatically by dropping the pyrolysis cup into the pyrolysis furnace (350°C). The vertical micro-furnace pyrolyzer was interfaced to a Varian 3900 GC equipped with a Saturn 2100T mass spectrometer (EI mode). A DB-5HT Inferno capillary column (30 m, 0.25 mm id and 0.10 μm film thickness) was used. The GC injector was set at 340°C and the MS interface at 300°C . The column flow was 1 ml min^{-1} and split flow 10 ml min^{-1} . Oven temperature was 100°C for 5 min, then increased by $20^\circ\text{C min}^{-1}$ to 350°C , and finally held for 1 min. Mass spectra scans ranged from 60 to 650 amu.

5.2.5 Methylation using TMS-diazomethane

TMS-diazomethane, a methylating reagent, was used to determine if the observed products of thermochemolysis were from simple methylation of methanol extractives of plant material. If there were no products after treatment with TMS-diazomethane, then it was assumed that they were actual thermochemolysis products requiring thermal hydrolysis conditions. For methylation using TMS-diazomethane, 500 µg of sample was dissolved in 30 µl of methanol and 40 µl of TMS-diazomethane was added to the vial. The sample was sonicated for 30 min at ambient temperature and a 1 µl aliquot of the final solution was injected into the GC injection port (Shadkami et al., 2009).

5.3 Results and Discussion

5.3.1 Optimization of thermochemolytic reaction using a direct thermochemolysis solids injector

The thermochemolytic reaction between kraft lignin and TMSH was optimized. Thermochemolysis products (Fig. 5.4) were compared at three different temperatures (i.e. 250°C, 300°C and 350°C) and the highest yield of high molecular weight products was at 350°C. However, the chromatograms were not significantly different. Figure 5.5 and 5.6(a) show the chromatograms at 250°C and 350°C, respectively. Furthermore, changing the amount of reagent from 5 µl to 50 µl for a 500 µg of a sample only caused the dilution of the solution and decreased the sensitivity. The split flow was also adjusted

to 5, 10 and 15 ml/min. A split flow of 10 ml/min was found to be optimal, providing adequate sensitivity while keeping column contamination at a minimum.

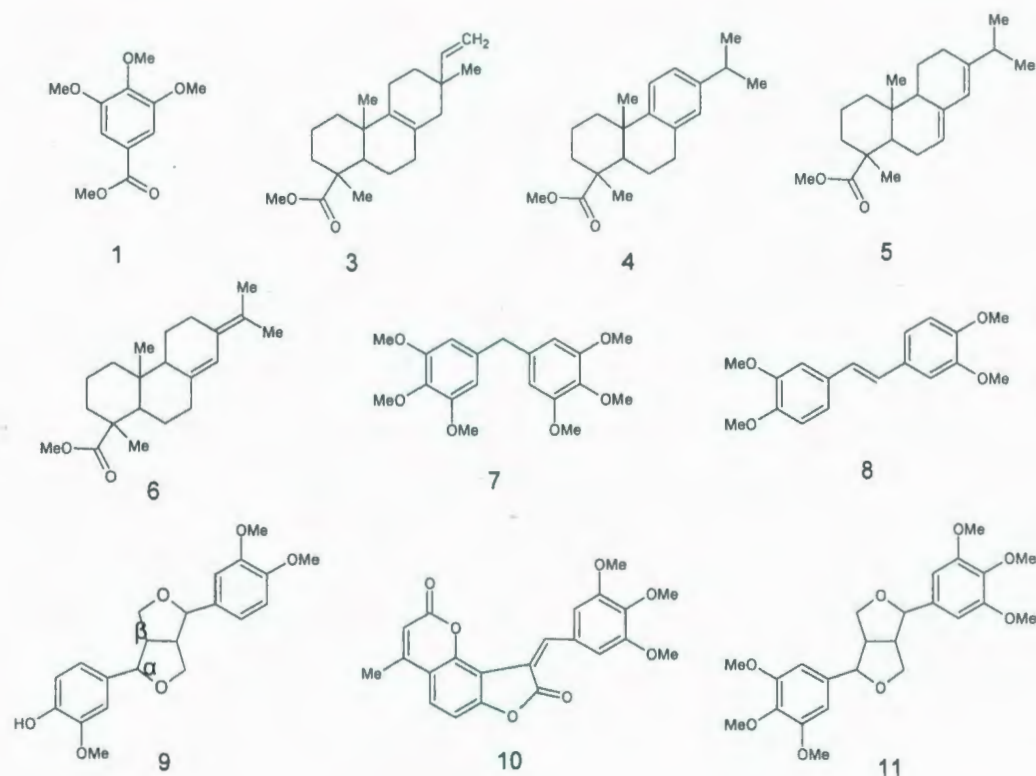


Fig. 5.4 Structures of thermochemolysis products identified by thermochemolysis-GC/MS as listed in Table 5.1.

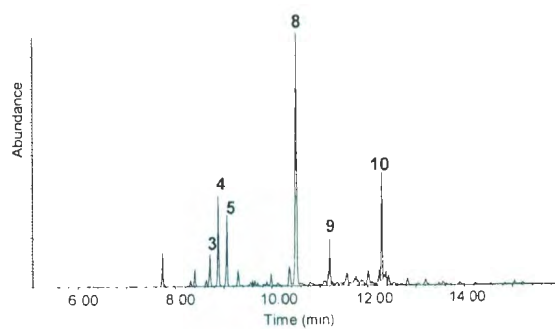


Fig. 5.5 Partial total ion chromatogram obtained from thermochemolysis (in presence of TMSH) kraft lignin (Indulin AT) at 250°C using a direct solids injector.

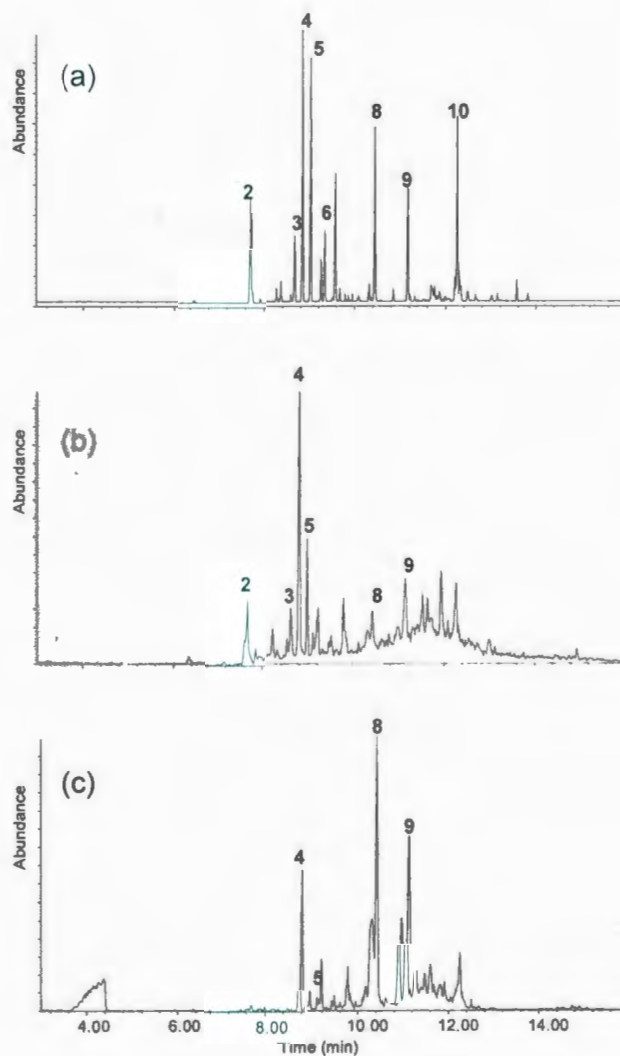


Fig. 5.6 Partial total ion chromatograms obtained from thermochemolysis (in presence of TMSH) of softwood lignins (a) kraft (Indulin AT), (b) lignosulfonate and (c) Tembec at 350°C using a direct solids injector.

5.3.2 Comparison of a direct thermochemolysis device vs a microfurnace pyrolyzer for thermochemolysis of lignin in the presence of TMSH.

As shown in Figure 5.7, the thermochemolysis products of softwood (Fig. 5.7a) and hardwood (Fig. 5.7b) lignins using a microfurnace pyrolyzer is similar to products of softwood (Fig. 5.6) and hardwood (Fig. 5.8) lignin using a direct thermochemolysis solids injector.

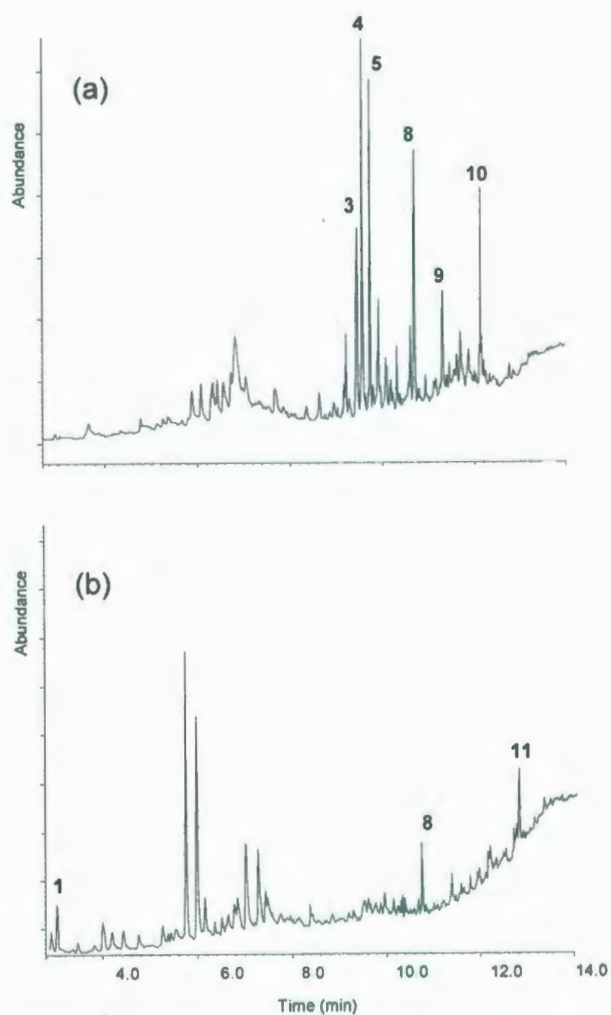


Fig. 5.7 Partial total ion chromatograms obtained from thermochemolysis (in presence of TMSH) of (a) softwood kraft lignin (Indulin AT) and (b) hardwood lignin using a microfurnace pyrolyzer.

5.3.3 Comparison of TMSH vs TMAH in thermochemolysis of softwood lignins.

Thermochemolysis of softwood kraft lignin (at 350°C) in the presence of TMAH using a microfurnace pyrolyzer yielded mainly 3,4-dimethoxystyrene, 3,4-dimethoxybenzaldehyde, 3,4-dimethoxybenzoic acid methyl ester and 3,4-dimethoxy cinnamic acid methyl ester (chromatogram not shown), which have also been reported as common thermochemolysis products of softwood lignins (Fig. 5.2) (Sithole, 2000). The presence of two methoxy groups in an aromatic ring suggested that these products were mainly guaiacyl derivatives resulting from cleavage of β -O-4 bonds in the lignin polymeric structure. Dominant peaks for guaiacyl derivatives and lack of peaks for syringyl groups also represented softwood thermochemolysis products because softwood contains more than 95% guaiacyl groups and 1% syringyl groups while hardwood contains 40-60% syringyl groups (Sithole, 2000).

Thermochemolysis using TMSH produced high molecular weight lignin markers (Fig. 5.4). This is in stark contrast to thermochemolysis using TMAH which produced lignin markers of smaller size, some representing monomeric units (Fig. 5.2). The advantage of dimeric markers, produced by thermochemolysis-TMSH, is that they uniquely represent part of the complex lignin structure.

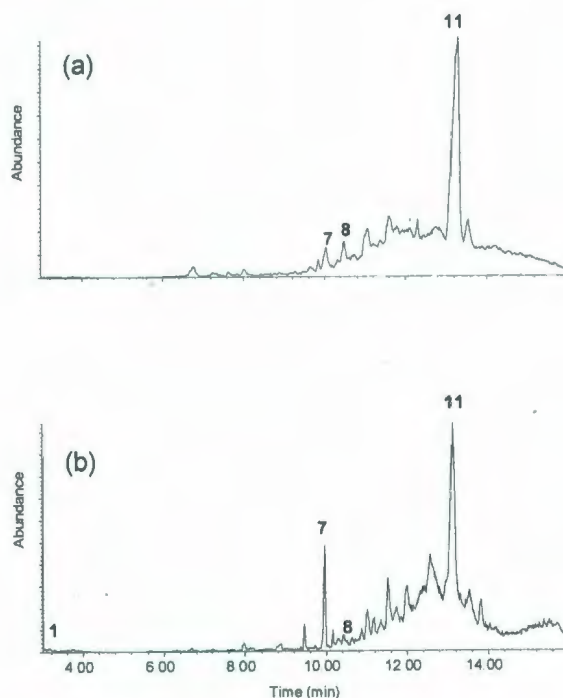


Fig. 5.8 Partial total ion chromatograms obtained from thermochemolysis (in presence of TMSH) of hardwood lignins (a) hardwood and (b) Alcell at 350°C using a direct solids injector.

The monomeric structures obtained by TMAH however, might be mistaken for markers of other classes of compounds such as condensed tannins.

There were also some useful similarities in structures of softwood lignin markers resulting from the above approaches. Sylvatesmin (9), a dimeric marker of softwood lignin, has two methoxy groups in their aromatic rings. Similarly, the monomeric markers produced from thermochemolysis of softwood lignin in presence of TMAH also

have two methoxy groups in their aromatic rings. As a result, both reagents produced guaiacyl derivatives from thermochemolysis of softwood lignins. In comparison to TMAH thermochemolysis products, the thermochemolysis-TMSH products (compounds 7, 8, 9, 10, 11 in Fig. 5.4) show that TMSH is a more selective and milder approach which allows for structural retention of the lignin subunits.

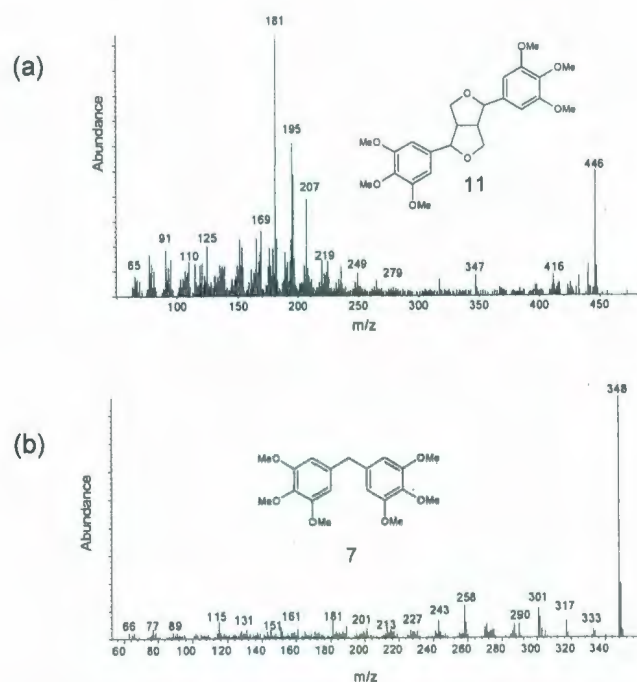


Fig. 5.9 EI mass spectra of (a) yangambin (11) and (b) Bis (3,4,5-trimethoxyphenyl) methane (7).

5.3.4 Thermochemolysis of different softwood lignins

Softwood lignins including kraft lignin, lignosulfonates (kraft lignin with a low amount of sulfonate) and Tembec lignin (extracted from a mixture of spruce, balsam and fir; Tembec is a trade name) were subjected to thermochemolysis in the presence of TMSH (350°C). Kraft lignin, a highly cross-linked polyphenolic, is the product of the kraft pulping process whereby extraction of lignin from wood is by alkali treatment (Alder, 1977).

As shown in Figure 5.4 one of the softwood lignin markers also known by the trivial name of sylvatesmin (9) (Miao et al., 2004), contains two attached tetrahydrofuran rings bonded to aromatic rings.

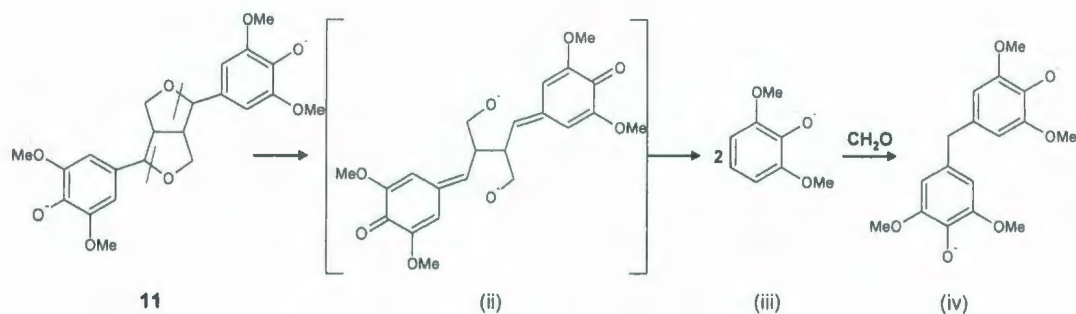


Fig. 5.10 Formation mechanism of compound (7), Bis (3,4,5 trimethoxyphenyl)methane, from Yangambin (**11**) during alkaline pulp process.

The fully methylated sylvatesmin is also called di-methylpinoresinol (Nakagawa-izumi et al., 2004). Sylvatesmin, a softwood thermochemolysis product, is proposed to exist as guaiacyl β - β subunits in the lignin polymeric structure. The product structure shown here would indicate that two of the phenolic groups from each aromatic ring were originally attached to lignin and cleaved during the thermochemolysis process.

Thermochemolysis of softwood lignins (Fig. 5.6) also produced compound **8**, 3,3',4,4'-Tetramethoxystilbene, which was previously observed in another thermochemolysis study involving lignin (Ishida et al., 2007). It was suggested that, for

this compound, two of the phenolic groups from each aromatic ring were bonded to a lignin polymeric structure. Compound **8** was also studied as a model belonging to a family of dimeric lignin subunits (Evstigneyev et al., 2004). As shown in Figure 5.6, another product, compound **10**, is another lignin marker. It contains two lactone type structures (γ and δ) and a syringyl group. Lactone type structures were previously observed in lignin marker studies (D'Auria et al., 2002).

In Figure 5.6 (a, b), the fatty acid compound **2**, a product from thermochemolysis of kraft lignin and lignosulfonates, was also found by other workers in the thermochemolysis of triacylglycerides in leaf litter (Hermosin and Saiz-Jimenez, 1999). Also shown in Figure 5.6 are pimaric acid methyl ester (**3**), dehydroabietic acid methyl ester (**4**), abietic acid methyl ester (**5**), and neoabietic acid methyl ester (**6**), all very common resin acids found in softwoods (Hovelstad, et al., 2006). Except for neoabietic acid methyl ester (**6**), which was found only in kraft lignin, the other resin acids were common both in kraft lignin and lignosulfonates. For Tembec lignin, only dehydroabietic acid methyl ester (**4**) and abietic acid methyl ester (**5**) were observed.

Additional work was done using TMS-diazomethane which is a facile methylating reagent for carboxylic acids, phenolics and alcohols. No interfering artifacts in analyses with GC-MS were observed (Norio et al., 1981). All lignin samples were analyzed with TMS-diazomethane and no obvious products were observed. These results are important

because it shows that the resin acid products obtained under thermochemolysis were released from their bonded structure to lignin or from encapsulation within the macromolecular lignin structure. These findings also indicate that the observed fatty acids thermochemolysis products were not in free form, rather originally as triacylglycerides, and that thermochemolysis was required to cleave the ester bonds.

Table. 5.1. Chemical assignment of major chromatographic peaks

Peak # ^a	MW	RT(min)	Thermochemolysis Product ^b	Wood Source ^c
1	226	3.01	3,4,5-Trimethoxybenzoic acid, methyl ester	H
2	294	7.63	6,9-Octadecadienoic acid, methyl ester	S
3	316	8.67	Pimaric acid methyl ester	S
4	314	8.85	Dehydroabietic acid methyl ester	S
5	316	9.04	Abietic acid, methyl ester	S
6	316	9.26	Neoabietic acid methyl ester	S
7	348	9.95	Bis (3,4,5-trimethoxyphenyl) methane	H
8	300	10.45	3,3',4,4'-Tetramethoxystilbene	H&S
9	372	11.18	Sylvatesmin	S
10	394	12.26	9H-Furo[2,3-H]chromene-2,8-dione, 4-methyl-9-(3,4,5-trimethoxybenzylidene)	S
11	446	13.11	Yangambin	H

^a all compounds identified in NIST library except compound 10 which is tentative, ^b as shown in chromatograms, ^c hardwood (H) or softwood (S).

Reliable analysis of resin acids, fatty acids, and triacylglycerides is very important because they cause technical and economical difficulties in the pulp and paper industry. The most common ingredients of pitch deposit are resin acids, fatty acids and triacylglycerides (Gutierrez et al. 2001).

5.3.5 Thermochemolysis of different hardwood lignins

Two hardwood lignin samples were subjected to thermochemolysis in the presence of TMSH. The highest molecular weight hardwood lignin marker was found to be yangambin (11) with a similar structure to sylvatesmin except that it has three methoxy groups in each aromatic ring (Fig. 5.4). The mass spectrum of yangambin is shown in Figure 5.9a. Yangambin has also been proposed to exist as syringyl β - β subunits attached to the three-dimensional lignin structure through phenolic groups from each ring (Nakagawa-izumi, et al., 2004). The present study confirms the difference between the guaiacyl and syringyl content of softwoods and hardwoods. In general, hardwoods contain 40-60% syringyl groups whereas softwoods contain 95% guaiacyl groups and 1% syringyl groups (Sithole, 2000). Although yangambin was suggested as a thermochemolysis product of lignin model compounds (Nakagawa-izumi et al., 2004), this is the first study that has reported yangambin as a thermochemolysis product of real lignin samples.

The other hardwood thermochemolysis product (7), bis (3,4,5-trimethoxyphenyl) methane, was likely produced from yangambin during the alkaline pulping process. To illustrate this, Figure 5.10 shows the formation mechanism of compound (7). This involves the opening of the furan rings followed by the formation of intermediate (ii) and, finally, the formation of compound (7, iv) in the presence of formaldehyde (Gierer and

Smedman, 1971). The mass spectrum of bis (3,4,5-trimethoxyphenyl) methane is shown in Figure 5.9b. Another thermochemolysis product of hardwoods is methylated gallic acid (1), commonly observed in other studies.

5.4 Conclusions

This study has advanced the use of thermochemolysis in the presence of TMSH using a direct thermochemolysis solids injector for the analysis of softwood and hardwood lignins by producing unique lignin markers. Softwood and hardwood lignins can easily be differentiated using a simple and economical direct thermochemolysis solids injector. This experimental approach revealed the presence of yangambin (containing syringyl groups) in hardwood lignins and the presence of sylvatesmin (containing guaiacyl groups) in softwood lignins. A common marker for both softwood and hardwood lignin was 3,3',4,4'-tetramethoxystilbene was also observed. The fact that only these lignin markers were observed in the chromatogram reveals that these compounds are dominant or they are more responsive to thermochemolysis using TMSH. This is the first study in which TMSH has been used in the characterization of lignin. Compared to TMAH, the reagent TMSH produced higher molecular weight lignin markers.

5.5 Acknowledgments

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Chapter 6: Conclusions

6.1 Concluding remarks

This thesis has advanced knowledge in thermochemolysis by developing an improved thermochemolysis technique (Chapter 2 and 3), constructing a novel thermochemolysis device (Chapter 4), and applying the improved techniques/devices in rapidly analyzing plant biomaterials (Chapter 2-5). Thermochemolysis is a unique *in situ* technique for analysis of biomaterials in plants. It involves the derivatization of analyte functional groups leading to improvements in the chromatographic behavior of analyte molecules so suitability for GC-MS analysis. In addition, thermochemolysis accentuates a mild thermal fragmentation of biomaterials and selective cleavage of ester and ether bonds rather than a harsh thermal fragmentation, which is the case in the traditional pyrolysis. Thermochemolysis also improves the characterization of polymeric structures such as lignins (Chapter 5) and condensed tannins (Chapter 3) by cleavage of ether and interflavonoid bonds, respectively. In most cases no sample extraction or work up is needed and solvent interaction is minimal because it can be purged off in the early stages of the thermochemolysis process. It is also very rapid and only small amounts of sample are required.

In this work, the application of TMSH in thermochemolysis was demonstrated in a wide range of biomaterials and the behavior of TMSH were evaluated. Until the present, TMSH had been mainly used for analyzing lipid compounds [1-6]. It was

revealed that TMSH is more suitable than TMAH, the most common thermochemolysis reagent, for analyzing thermally-sensitive compounds such as certain polyphenolic structures that required lower thermochemolysis temperatures. Products derived from thermochemolysis of catechins in presence of TMSH are intact unique catechin products that represent the parent macromolecule (Fig. 3.6). Thermochemolysis in the presence of TMAH, however, yields excessively fragmented products (Fig. 1.6). Another example of the milder behavior of TMSH can be observed by comparison of lignin thermochemolysis products in presence of TMAH (Fig. 1.8) and TMSH (Fig. 5.4). The reagent TMSH allows for the structural retention of the lignin subunits.

Up to this study, TMSH was known for its behavior in the cleavage of ester bonds in triacylglycerols. The present study was the first to show that TMSH is able to cleave other bonds including condensed tannins interflavonoid bonds (terminal units only) and lignin ether bonds. The main disadvantage of TMSH is that the aqueous samples must be dried prior to analysis as TMSH is moisture sensitive.

The research of this thesis has clearly shown that *in situ* thermochemolysis of biomaterials is suitable for "whole sample" chemical profiling (WSCP). WSCP refers to analyzing a wide range of components using small amounts of

sample (with varying complexity) without any sample preparation. Among different tannin samples, the analytical approach exhibited a broad range of GC products from small phenolic compounds, i.e., 1,3,5-trimethoxybenzene with a molecular weight of 168 amu, to very large condensed tannin dimmers with a molecular weight of a 570 amu. This product range covers almost the full mass range of GC-MS (profiled in Chapter 3). In the study of lignins, products were profiled from 3,4,5-trimethoxybenzoic acid, methyl ester (MW = 226 amu) to a lignin marker (MW = 394 amu) as illustrated in Chapter 4.

Chapter 2 is devoted to the thermochemolytic chemical profiling of secondary metabolites in pine needles of trees which were subjected to exposure to different ozone levels. In this study phenolic and diterpene resin acids in needles from ozone-tolerant and ozone-sensitive pine trees fumigated at different ozone levels were profiled by thermochemolysis GC-MS. The study in partnership with Forestry Canada, NB, was to examine if certain clones of white pine were more resistant to ground level ozone and to study the effects of ozone on tree metabolism. The first analytical result of direct needle analysis was that the resin acids anticopalic, 3-oxoanticopalic, 3 β -hydroxyanticopalic and 3,4-cycloanticopalic acids were prominent in the ozone-sensitive pine; however, only anticopalic acid was present in the ozone-tolerant clone. It was also shown that the concentrations of 3-hydroxybenzoic and 3,4-dihydroxybenzoic

acids decreased with increased ozone dosage. It was likely that these two phenolic acid thermochemolysis products originate from a shikimate pathway that produces aromatic-containing secondary metabolites. This study has shown that the applied thermochemolysis technique will be useful in biomonitoring of ozone levels based on phenolic acid concentrations.

The idea behind the investigation involving the analysis of catechins and condensed tannins by thermochemolysis and two-step methylation method (Chapter 3) originated from a single experiment wherein the thermochemolysis of catechins in the presence of TMSH surprisingly produced an intact, partially methylated catechin molecule. The GC retention time of this compound was significantly higher than those of the small phenolic compounds that were expected to be observed, based on previous literature studies. To be able to observe an intact catechin is an advantage because intact catechin molecules will uniquely represent the original tannin macromolecule (Fig. 3.6). Other studies only obtained small phenolic molecules which result from extensive bond cleavage within the catechin molecule when TMAH and higher temperatures were used (Fig. 1.6).

In further investigations, the epimerization behavior of catechins was observed wherein epicatechin, its *cis* epimer, was formed and observed in GC/MS when treated under thermochemolysis using alkaline conditions. Experiments using the

standard B1 dimer, a condensed tannin dimer, were able to produce methylated catechin, thus revealing the composition of its terminal unit. Another study [7] indicated that the terminal unit retains its chemical structure under based-catalyzed conditions. Finally, the polymeric structure of condensed tannins was further explored using a novel two-step methylation technique (TMS-diazomethane followed by thermochemolysis). The pre-methylation step in the analysis allowed for the observation of dimer catechin at MW = 540 amu. It must be noted from further GC work, that this large, methylated marker was only observed if a high temperature GC column was used since it eluted at an oven temperature of 375°C. The formation of this dimer assisted in the verification of the condensed tannins under study containing catechin monomeric units.

Chapter 4 reports the fabrication of a direct thermochemolysis solids injector for use in GC injection ports and the application of the device in the thermochemolysis of a wide range of plant biomaterials. As expected, it was observed that the use of the solids injector has a significantly greater ability to detect high molecular weight products than using the common microfurnace pyrolyzer because of the close proximity of solids injector/sample to the column. Traditional pyrolyzers can suffer from discrimination of high molecular weight compounds in transferring products from the pyrolyzer to the GC injection port [8].

Initially, the analytical usefulness of this thermochemolysis device was tested with a microfurnace pyrolyzer and it exhibited similar chromatographic results for the thermochemolysis of standard catechin. Further, it was shown that this solids injector was suitable for real sample analysis containing a wide range of compounds including small phenolics, fatty acids, diterpene resin acids and flavonoids. For most biomaterials, minimal sample preparation is required. An example of real sample applications is the analysis of St. John's Wort, an herbal medicinal plant. The direct analysis of a small portion of the medicine tablet resulted in a clear compositional profile of a number of fatty acids and a series of unique (and intact) flavonoids.

Chapter 5 further explores the use of direct thermochemolysis solids injection for the chemical analysis of lignin macro-molecules. This research was the first to observe new dimeric thermochemolysis products of which these two lignin markers can be used to differentiate hardwood lignins from softwood lignins. The structures of the dimeric markers (Fig. 5.4) support the structures of previously proposed dimeric model substructures (Fig. 1.7). Since they are dimeric in structure, they more uniquely represent lignins than the smaller products previously found as monomeric structures (Fig. 1.8). It is not clear why only tetrahydrofuran type substructures were produced in this study. The dimeric products, sylvatesmin and yangambin, may represent an

abundant lignin substructure or they were more clearly observed because they are more easily released (compare to other substructures; Fig. 1.7) under thermochemolysis conditions.

Some limitations with thermochemolysis remain. The choices of *in situ* derivatization reagents are limited. They are commonly organic bases and are not suitable for the analysis of base-sensitive compounds. Each project (each Chapter) had its limitations. In Chapter 3, the higher the average DP of tannins, the less likely dimer markers will be observed by two-step methylation GC/MS. As well, direct thermochemolytic analysis identified only the terminal units of the tannin polymers. However, the studies using pre-methylation have been shown to stabilize the base-sensitive tannins and allow for detailed analysis of the complex stereochemistry of monomeric units and of interflavonoid linkage. In Chapter 4, it is noted that several usages of the direct thermochemolysis solids injector causes septum bleeding. Another limitation of thermochemolysis is for analysis of small phenolic compounds with more than one hydroxyl group. Thermochemolysis products usually contain both partially and fully methylated phenolic compounds as in the case for the thermochemolysis of gallic acid standard. However, it was not the case for gallic acids present in plant samples, probably because gallic acid was bonded to other compounds and there was enough time to fully methylate the phenolic acid before

volatilization. A challenge in future thermochemolysis studies is that of exploring thermochemolysis mechanisms, not only with simple standards, but within real sample bio-matrices.

6.2 Ideas for future work

Organic synthesis: A novel idea is to adapt the thermochemolysis concept such that the cup (i.e. the sample holder) in a microfurnace pyrolyzer is used as a reactor vessel under He inert gas to maintain organic synthesis reactions for rapidly reacting reactants at medium to high temperatures. The environment will be inert and only a very small amount of sample is required. The pyrolysis unit has the added benefit to be able to analyze the products via GC/MS or directly by MS.

Pre-treatments in thermochemolysis: In Chapter 3 a two-step methylation technique (TMS-diazomethane followed by thermochemolysis) was developed which could be modified to involve silylation followed by thermochemolysis in presence of TMSH. The two-step methylation study produced dimer markers. It would be expected that silylation followed by TMSH thermochemolysis could produce dimer and even trimer markers with different molecular weights and

structures. Silylation will also derivatize alcoholic groups as well the acidic functional groups with TMSH methylates.

Other thermochemolysis reagents: Until this research was undertaken the scope of thermochemolysis has been mainly limited to using TMAH. The present study has advanced thermochemolysis by the extensive use of TMSH; however, other reagents can be used including the reagent, dimethyl carbonate. This reagent was recently proposed as a thermochemolysis reagent for pyrolysis transmethylation of triacylglycerols at 700°C [9]. It has not yet been applied to the analysis of other compounds of interest in this present study.

The chemistry of TMSH explored: The present results of this thesis have clearly shown the usefulness of TMSH as the thermochemolysis reagent of choice. But information about the chemistry of TMSH is very limited and in some cases misleading. For example, in two articles the pK_b of TMSH is reported to be 12 which is wrong [6,10]. It has always been assumed that the mechanism of methylation by TMSH is identical to that of TMAH. An in-depth study of its real mechanism may reveal certain details which can be used for reaction/derivatization selectivity.

Biomaterial complexity: The interaction of compounds with each other in plant

biomaterials under thermochemolytic conditions is not clear. Most studies have focused on finding the best technique for the analysis of a group of compounds or series of compounds, but there are limited works that help explain how compound will behave when initially bonded to other compounds. For example, resin acids have always been known as extractives, components that can easily be solvent extracted from wood. This thesis showed evidence that the resin acids present after pulping process are non-extractives and maybe bound to the lignin. Based on the results of the TMSH thermochemolysis conditions they seem to be originally bound to the lignin macrostructure. This is important because these strong compound-compound interactions might influence the properties of the finished products.

Quantitative analysis: One of the weaknesses of thermochemolysis studies is the limited number of publications involving quantitative analysis [11]. The major requirements for quantitative analysis are reproducibility, standard curves, and dynamic range. Statistical analysis is needed to evaluate reproducibility of data and to investigate how the mean of variables are affected by different types and combinations of instrumental factors. Future studies in the quantitative analysis accentuating relevant statistical analysis are needed.

6.3 References

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